THE UNIVERSITY *of* EDINBURGH

Molecular and antigenic characterisation of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks and *in vitro* cultures

Milagros Postigo

PhD thesis-The University of Edinburgh-2006

Table of contents

Declaration

The work presented here is all my own except where it is indicated and the composition of the thesis was completed by myself

Maria Milagros Postigo Mercades

Dedication

Dedico esta tesis a mis padres, Eduardo y Luisa; y a mi querida niña Camila, para que este logro sirva en un futuro como ejemplo de perseverancia.

Acknowledgements

I would like to express my gratitude to my supervisors: Keith Sumption and Sue Welburn for giving me the opportunity to undertake this project; Frans Jongejan, for making me welcome in Utrecht University and for your total support that enabled me to complete my work; and Ivan Morrison for being agreeable when I needed to continue with my studies abroad and for your support with the writing of my thesis. To all, thanks for the guidance throughout my research.

My heartfelt thanks to Lesley Sakyi, for your unconditional support, excellent scientific and technical advice, fruitful discussions, encouraging words, and for all the written English corrections which made my life much easier during all these years. Many thanks for your invaluable friendship.

I would like to thank all the staff and students at CTVM, especially many thanks to Frank, Alison and Tim, for being such good friends and supporting me a lot in the lab when I needed it most (Dorothy is also grateful!); Olga, Karine, Alan Walker, Sandra Grant, Wilma Robertson and Ann Morrison for being good company and always having encouraging words for me. Jeanette MacDonald and Pauline McManus are thanked for helping me with administrative issues; Paul Wright for his help in the animal house and Stuart Lansley for helping me with computing issues.

I would like to extend my gratitude to all the staff and students at Utrecht University, especially to Cornelis, my most sincere thanks for being always there and for guiding me well in the heartwater research; to Omar, many thanks for being so kind, helpful, and a good friend and for all your invaluable support and patience!!! during the transmission experiments; to Ana, Frits, Frans Kooyman and Erik, for willingly teaching me all the tips and tricks in the lab and for the excellent scientific support; Patricia, Wim and Frans are thanked for their support in the animal house and company during the weekends!; to Ella and Hans, for helping me with all the extra-academic issues; Maria and Anton thanks for your friendship and for making easier my adaptation to The Netherlands; to Ard, Radi and Zorica for always helping me every time I bother you!. My thanks to all of you for making the work environment very pleasant.

To Alan Walker, Dominique Martinez, Tracey Coffey, Agustin Estrada-Pena and Stuart Smith, for providing me with material and/or useful advice and to the previous and current heartwater projects (European Union projects ICA4-CT-2000-30026 and FP6-003713), for supporting my research.

To my former teachers and colleagues at the “Instituto Venezolano de Investigaciones Cientificas” (IVIC), for all the valuable lessons taught during the first years of my research career.

And last but not least, to my family for endless encouragement: Primero que nada a ti Camila, por la comprension y paciencia que has tenido con mami a pesar de tu corta edad; gracias papa y mama por enseñarme a luchar por lo que se quiere y animarme a ampliar mis horizontes, este logro se los debo a Uds.!. A mi queridisima tia Esther, la mejor nanny del mundo!, gracias por tus oraciones y por apoyarnos cuando mas lo necesitamos; a abu Alicia por apoyo moral, y a mi amadisimo Carlos, gracias por tu amor, por tu apoyo incondicional, por tu enorme paciencia y por ser el mejor compañero de vida que he podido tener, definitivamente no hubiese logrado esta meta sin ti.

Gracias a Dios y a la Virgen por darme la fuerza para continuar.

Abstract

The rickettsial pathogen *Ehrlichia ruminantium*, transmitted by ticks of the genus *Amblyomma*, causes heartwater, an economically important, often fatal disease of domestic and wild ruminants in sub-Saharan Africa and in the Caribbean. The studies described in this thesis have contributed to understanding several aspects of heartwater. First, a real-time PCR method was developed in order to study the kinetics of infection with *E. ruminantium* in the mammalian host. The assay was validated for specificity and sensitivity and was used to estimate numbers of the organisms in the blood of infected sheep. However, organisms were only detected during the clinical phase of infection, indicating that the way in which it was applied did not provide sufficient sensitivity to follow the early stages of infection. This PCR assay was then used, together with transcription and proteomic analyses, to investigate differential gene expression of *E. ruminantium* in the arthropod and mammalian hosts, in order to identify genes that may allow the organisms to successfully adapt to different environments. These studies used *in vitro* tick and mammalian cell culture systems, as well as tissues from infected *A. variegatum* ticks, and initially focused on the *map1* multigene family. Although transcripts for most of the *map1* paralogs were detected in organisms grown *in vitro*, in both mammalian and tick cells, only transcripts from *map1* and *map1-1* were detected in infected ticks. Moreover, *map1-1* transcripts were more abundant in midguts than in salivary glands whereas *map1* transcripts were most abundant in salivary glands and were expressed at higher levels following several days of tick feeding on a mammalian

host. Because of the quantities of material required, proteomic analysis was only possible using *in vitro*-cultured organisms. Comparison of proteins encoded by the *map1* cluster in *E. ruminantium* grown in tick or bovine endothelial cell cultures, using 2D gels and MALDI-TOF analysis, revealed that different proteins predominated in the corresponding spots in 2D gels from the different cultures; products of the *map1-1* gene were abundant in tick cells, while products of *map1* were abundant in endothelial cells. The detection of higher levels of *map1* transcripts in salivary glands than in midguts of infected ticks, together with the presence of abundant MAP1 protein in organisms grown in mammalian but not in tick cell lines, suggest that expression of this protein may be associated with infectivity for mammalian cells. In contrast, *map1-1* transcripts were abundant both in midguts of infected ticks and in tick cell lines, and the protein was expressed at high levels in infected tick cell cultures. Since both of these stages have low infectivity for sheep, these results suggest that the MAP1-1 protein may play an important role within the vector, possibly associated with colonisation and replication of *E. ruminantium* in the tick midgut. Collectively these findings suggest that this multigene family is involved in functions of biological relevance in different stages of the life cycle of *E. ruminantium*. Lastly the suppression subtractive hybridisation (SSH) technique was applied to RNA extracted from *E. ruminantium*-infected endothelial and tick cell cultures in an attempt to sample a large portion of the *E. ruminantium* genome for differentially expressed genes; although not resulting in identification of any differentially transcribed genes in the present study, this method was shown to work in principle.

Chapter 1: Literature review

1.1 General introduction

Heartwater or cowdriosis is an important, often fatal, tick-borne disease of domestic and wild ruminants that occurs in sub-Saharan Africa and also on some Caribbean islands. There is also concern that the disease could spread to the American continent (Uilenberg, 1983). Imported breeds of cattle, sheep and goats are highly susceptible, but indigenous populations in endemic areas are often resistant to infection. Therefore, heartwater is a major obstacle to the introduction of highly productive animals into endemic areas and also it is a major disease problem when local animals are moved from heartwater-free to heartwater-infected areas (Camus et al., 1996). For instance, the total economic losses directly associated with heartwater have been estimated as US$ 5.6 million per year for cattle in Zimbabwe (Mukhebi et al., 1999), and US$2 million per year for cattle and goats in Guadeloupe (Camus et al., 1996).

The causative agent of heartwater is the rickettsial pathogen *Ehrlichia* (previously *Cowdria*) *ruminantium* which is transmitted by ticks of the genus *Amblyomma* (Bezuidenhout, 1987).

E. ruminantium is a gram-negative obligate intracellular bacterium, unique among other related pathogens of the family *Anaplasmataceae* because of its tropism in the vertebrate host for vascular endothelial cells. It has also been found in neutrophils, macrophages and reticulo-endothelial cells (Prozesky and Du Plessis, 1987). In ticks, *E. ruminantium* infects most tissues and has been shown to replicate in midgut epithelial cells and salivary glands (Kocan and Bezuidenhout, 1987). These organisms enter cells by a process resembling phagocytosis and multiply within a

parasitophorous vacuole, avoiding normal phagocytic degradation. Different developmental forms, including elementary, intermediate and reticulate bodies, have been identified at different stages of infection and in different cells (Prozesky and Du Plessis, 1987). However, the precise sequence and biological relevance of these developmental forms during infection *in vivo*, in vertebrate and tick hosts, and for the tick-host-pathogen interactions are poorly understood.

1.2 E. ruminantium: definition and classification

The causative agent of heartwater is an obligate intracellular, pleomorphic bacterial pathogen, originally described by the microbiologist E. V. Cowdry as *Rickettsia ruminantium* (1925a, b). It was later renamed *Cowdria ruminantium* in honour of Cowdry (Moshkovski, 1947) and recently reclassified as *Ehrlichia ruminantium* (Dumler et al., 2001). The current classification of the organism is based on phylogenetic analysis of sequence similarity in the 16S rRNA gene and in the gro*ESL* gene sequences. Within the *Ehrlichia* genus *E. ruminantium* is considered to be most closely related to *E. canis* and *E. chaffeensis* (Dumler et al., 2001). A similar close relationship between these *Ehrlichia* species was found by Taillardat-Bisch et al. (2003), Brayton et al. (2005) and Hotopp et al. (2006), based on sequence similarity of the *rpoB*, 16S rRNA and *omp* genes respectively. The genera *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* form the family *Anaplasmataceae* in the order *Rickettsiales* (Fig. 1.1).

The recent publication of complete genome sequences for *E. ruminantium* (Collins et al., 2005), *Wolbachia pipientis w*Mel (Wu et al., 2004), *W. pipientis w*Bm (Foster et al., 2005), *Anaplasma marginale* (Brayton et al., 2005), *Anaplasma* (previously *Ehrlichia*) *phagocythophilum*, *E. chaffeensis*, *E. canis* and *Neorickettsia sennetsu* (Hotopp et al., 2006), has allowed comparative genomic analysis that shows a conserved gene organisation between *E. ruminantium* and *A. marginale*, indicating that *Ehrlichia* and *Anaplasma* are indeed very closely related bacteria. By contrast, a lack of synteny was observed between the genomes of *E. ruminantium* and *Rickettsia* and *Wolbachia* species (Frutos et al., 2006).

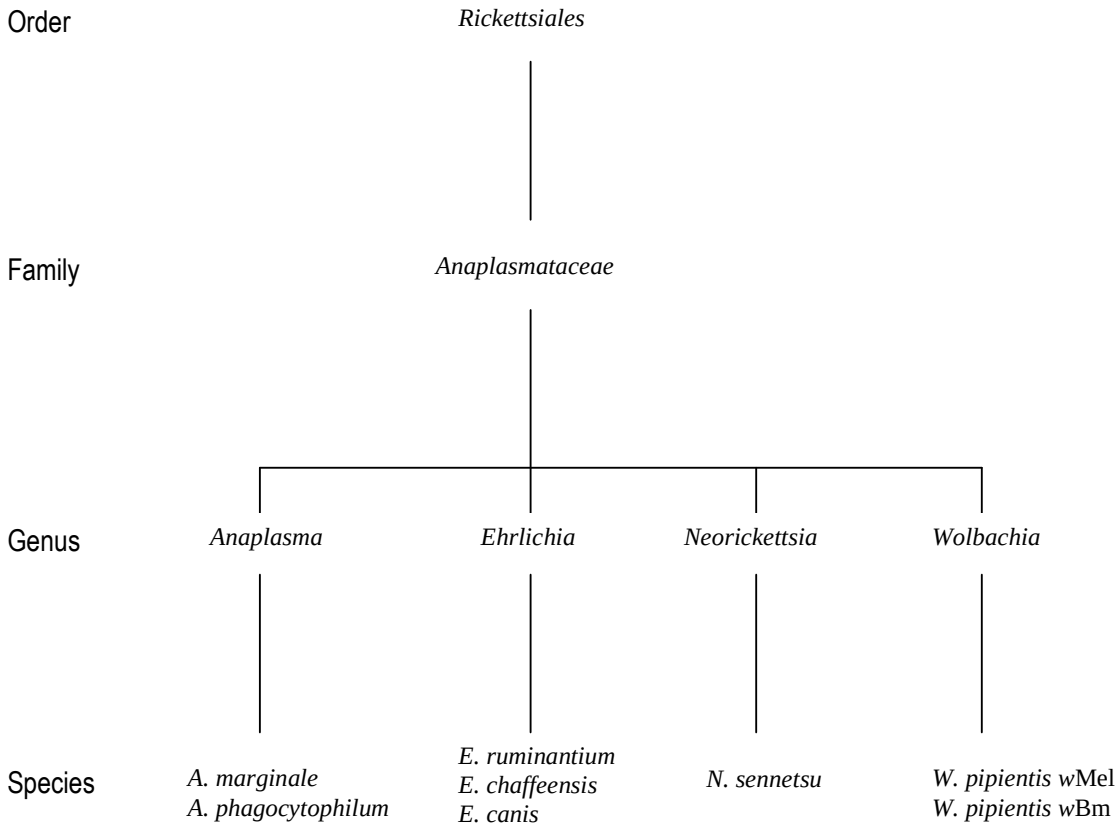


Fig. 1.1: Current classification of *E. ruminantium* in the family *Anaplasmataceae* (Order Rickettsiales) and examples of species in the different genera (Classification taken from Dumler et al., 2001).

1.2.1 Life cycle

E. ruminantium has a development cycle in which it alternately colonises a mammalian host and a tick vector. In the vertebrate host the spread of organisms from the site of the infection to the rest of the body is poorly understood. There appears to be an early phase of development in leucocytes (neutrophils and possibly also monocytes). Infection of these cells may occur at the tick bite site or in the regional lymph nodes following drainage of the organisms from the site of infection in afferent lymph (Du Plessis, 1970, 1975). Parasitised leucocytes then appear to drain into the general circulation. After multiplication in the leucocytes, organisms are released into the general circulation where endothelial cells lining blood vessels become infected. Another cycle of replication is completed in endothelial cells giving rise to colonies of organisms (Pienaar, 1970). After endothelial cells disrupt, numerous organisms, known as elementary bodies are released and a new infectious cycle is initiated in endothelial cells (Prozesky and Du Plessis, 1987). It is during this period that the organisms may be taken up by feeding ticks.

Prozesky and Du Plessis (1987) proposed that organisms initially develop and multiply in gut epithelial cells of ticks and subsequent stages invade and develop in the salivary glands. This is thought to be followed by transmission in saliva to the vertebrate host; however, the presence of mammal-infective organisms in tick saliva has not yet been confirmed. The detection of the organism in haemocytes suggested that *E. ruminantium* might spread within these cells in the haemolymph from the intestinal tract to other organs of the tick, including the salivary glands (Hart et al., 1991). However, the stage at which *E. ruminantium* migrates from midguts to

salivary glands remains uncertain. Yunker et al. (1993) detected *E. ruminantium* in salivary glands of unfed adults (infected as nymphs) of *A. hebraeum* using an *E. ruminantium*-specific DNA probe, demonstrating the early invasion of *E. ruminantium* in salivary glands. Fig. 1.2 shows a schematic representation of the life cycle of *E. ruminantium*.

E. ruminantium is an obligate intracellular pathogen that appears to require receptor-mediated endocytosis for infection of host cells. Members of the family *Anaplasmataceae* lack common pili or capsules; thus, these organisms are believed to bind to the host cells via their outer membrane proteins (Rikihisa, 2003). For instance, *A. phagocytophilum* uses P-selectin glycoprotein ligand 1 (PSGL-1) and α1,3-fucosylated, α2,3-syalylated glycans as a receptor-mediated pathway for cellular adhesion and entry to neutrophils, and a paralog of the P44 family of outer surface proteins may facilitate *A. phagocytophilum* binding to human PSGL-1 (Carlyon and Fikrig, 2003). After binding to their target receptor(s), the organisms enter the host cells in membrane-bound vacuoles (parasitophorous vacuoles) or inclusions, where they replicate, and the strategy for survival includes inhibition of phagosome-lysosome fusion (Weiss, 1991). Different members of the family use different strategies. When a human promyelocytic leukaemia cell line HL-60 is co-infected with *E. chaffeensis* and *A. phagocytophilum*, these bacteria enter into and retain separate inclusion compartments with different characteristics within the same cell. *E. chaffeensis* replicates in a vacuole containing early endosomal markers, but not lysosomal markers and inhibits the maturation of the endosome to evade destruction by lysosomal enzymes (Zhang et al., 2004b). *A. phagocytophilum* resides in a compartment that does not posses characteristics of either late or early

endosomes and avoids lysosomal fusion by isolating itself from host endocytic and exocytic vesicular traffic (Rikihisa, 2003; Carlyon and Fikrig, 2003). The mechanisms of intracellular survival of *E. ruminantium* have not been investigated.

Fig. 1.2: Schematic representation of the lyfe cycle of *E. ruminantium* (described in detail in Chapter 1, section 1.2.1), was kindly provided by Dr. L. Bell-Sakyi.

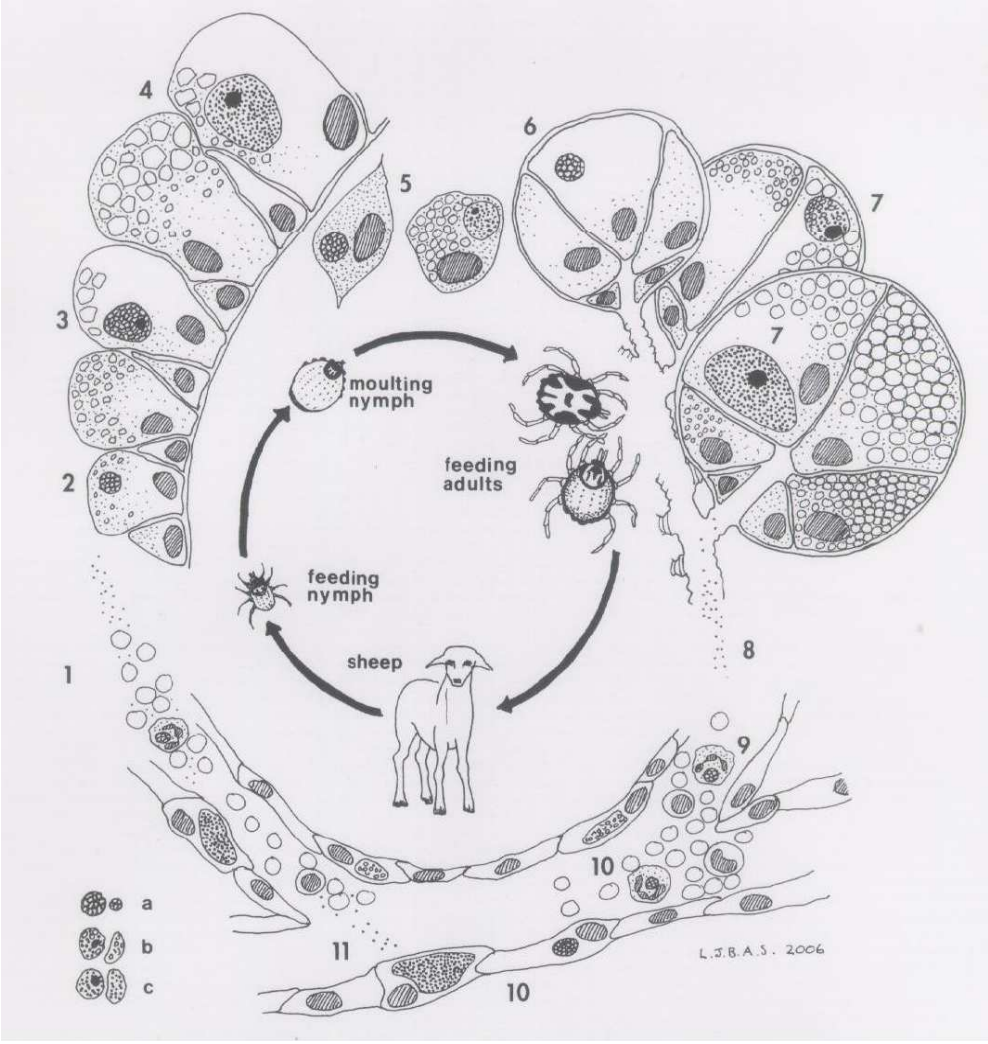


Fig. 1.2: Lyfe cycle (previous page):A larval or nymphal tick feeding on an infected host ingests *E.ruminantium* elementary bodies and possibly infected neutrophils (**1**). *E. ruminantium* infects and replicates in midgut epithelial cells where morulae containing tightly-packed reticulated (**2**, **3, a**) or electron-dense (**4**, **c**) bodies have been observed. *E. ruminantium* have been demonstrated in haemocytes (**5**), which may be how they are transported to the salivary glands. Eventually *E. ruminantium* infects and replicates in salivary glands where colonies of reticulated forms (**6, a**) and larger colonies containing electron-dense forms (***7***, **b, c**) have been observed. Transmission to the host takes place when infective forms of *E. ruminantium* are released from the salivary glands (**8**) and transferred, probably with the saliva, to a new vertebrate host during tick feeding. Several days later, *E. ruminantium*-infected neutrophils are observed in the general circulation (**9, a**), then *E. ruminantium* invades and multiplies in endothelial cells (**10 a, b, c**) in the lining of blood vessels. Eventually the endothelial cells disrupt and elementary bodies are released to the circulation (**11)** to infect more endothelial cells or to be taken up by feeding ticks.

1.2.2 Hosts and vectors

1.2.2.1 Hosts:

Heartwater affects domestic cattle (*Bos taurus* and *Bos indicus*), sheep and goats. Domestic Asian buffalo (*Bubalis bubalis*) are also susceptible and can die from heartwater. A large number of wild African and non-African ruminants as well as non-ruminant animals, including wild rodents and the laboratory mouse, the leopard tortoise, the guinea fowl and the ferret, are susceptible to infection with *E. ruminantium*, in some cases developing clinical signs (Oberem and Bezuidenhout, 1987).

Although wild animals have been implicated as reservoirs of infection, their importance in the epidemiology of heartwater is unclear (Camus et al., 1996). Antelopes appear to be important reservoirs, especially in cases of farms adjoining animal reserves. The leopard tortoise and crowned guinea fowl, which are asymptomatic or show only mild clinical signs, are potentially significant reservoirs, as *A. hebraeum* and *A. variegatum* commonly feed on these animals, whereas infection in wild rodents (rarely infected with *Amblyomma* ticks) is believed to be of little significance for livestock. Also, the infected tick, which is able to survive for 15 months and remain infected with *E. ruminantium*, and the domestic hosts, which become carriers of infection following recovery from primary infection, are themselves important reservoirs of infection (Camus et al., 1996).

Susceptibility to heartwater in domestic animals depends on a number of factors including breed, age, non-specific-resistance factors and immune status. a) Breed: In contrast to indigenous breeds of domestic ruminants in which mortality following infection with *E. ruminantium* is less than 5%, introduced exotic breeds

and their crosses are highly susceptible to the disease. Mortality levels of 60% and greater have been reported for Merino sheep and European cattle breeds (Camus et al., 1996). The innate resistance of indigenous breeds has not been investigated in detail and the mechanisms responsible for resistance are unknown. Comparison of resistance of Saanen (an exotic breed) and South African indigenous goats and F1 crosses between the two breeds demonstrated that the resistance exhibited by the indigenous goats was dominant in the crossbred offspring (Yunker 1996). b) Age: Very young animals are resistant to disease. This resistance appears to be independent of the immune status of the dam, and therefore should not be confused with passive immunity acquired through the colostrum. This period of resistance is short-lived in lambs (1-9 days) and longer in cattle (3-4 months). c) Non-specific resistance: In cattle over one year of age, a link between resistance and high levels of the serum protein conglutinin has been reported. However, the mechanism of this effect is unknown (Du Plessis and Malan, 1987). d) Immune status: Following recovery from infection, ruminants acquire protective immunity which, in sheep, has been reported to last for periods ranging from six months to 5 years, and in cattle, for at least two years in the absence of ticks. The degree of immunity is not related to the severity of the disease prior to recovery, and recovered animals can contract the disease two or three times in a single year, occasionally with a fatal outcome. These relapses are more frequent in cattle (Camus et al., 1996). Antibody levels in ruminants that recover from the disease do not correlate with protection or duration of immunity, which appears to be mainly cell-mediated and linked to the persistence of the pathogen in the tissues (Totté et al., 1999).

1.2.2.2 Vectors:

Ticks of the genus *Amblyomma* (Acari: Ixodidae) are the only proven vectors of *E. ruminantium*. These three-host ticks are widespread, although species proven to be field vectors of heartwater are all of African origin. Of these, *A. variegatum* is the main vector species throughout most of sub-Saharan Africa and the only vector in the Caribbean. *A. hebraeum* replaces *A. variegatum* in Southern Africa, and *A. astrion*, *A. cohaerens*, *A. gemma*, *A. lepidum* and *A. pomposum* are secondary in importance because of limited distribution or infrequent parasitism of domestic stock. A third category, referred to as accidental vectors, consists of species that do not normally feed on domestic stock: *A. marmoreum*, *A. sparsum* and *A. tholloni*. In the American mainland, three potential (experimentally proven) vectors are known: *A. cajennense*, *A. maculatum* and *A. dissimile* (Camus et al., 1996). Attempts to transmit heartwater using ticks of other genera such as *Rhipicephalus evertsi*, *Boophilus decoloratus*, *R. appendiculatus*, *Hyalomma truncatum*, and the soft tick *Ornithodorus savignyi* were not successful (Camus et al., 1996).

Amblyomma spp. have a wide host range, especially in the immature stages. Larvae and nymphs feed on small mammals, ground-feeding birds, reptiles, cattle, sheep and goats. Adult ticks prefer cattle, but can also be found on sheep, goats, horses, camels, dogs and some large wildlife. Wooded savannah or bushland are the habitat requirements of most *Amblyomma* spp. None of the vector species survive when rainfall is less than 250 mm or more than 2,800 mm.

The transmission of heartwater by ticks is transstadial, i.e. from larvae to nymph, from nymph to adult and from larvae through nymph to adult (even if the

nymph feeds on a non-susceptible animal). Intrastadial transmission is possible; an infected male may become infected and transfer to another host to which the infection can be transmitted (Andrew and Norval, 1989b). Repeated transmission to successive hosts by males of *A. variegatum*, infected as immature instars, has also been observed (Camus et al., 1996). Although there is one report of transovarial transmission (Bezuidenhout & Jacobsz, 1986), this route of infection is thought to be not important. A single infected nymph or a single infected male or female can transmit the disease (Camus et al., 1996). Transmission of the infection by ticks requires a delay after attaching to a susceptible animal; between 27 and 38 hours in nymphs and between 51 and 75 hours in adults (Bezuidenhout, 1987). Fig. 1.3 shows different stages of *A. variegatum*.

A

B

C

Fig. 1.3: A. variegatum female (left) and male (right) (A); *A. variegatum* mating on the host, note females at different stages of feeding (B); egg laying in *A. variegatum*, fully engorged female in the centre and fed male on the left are shown for comparison (C). (These pictures were kindly provided by Dr. Alan Walker).

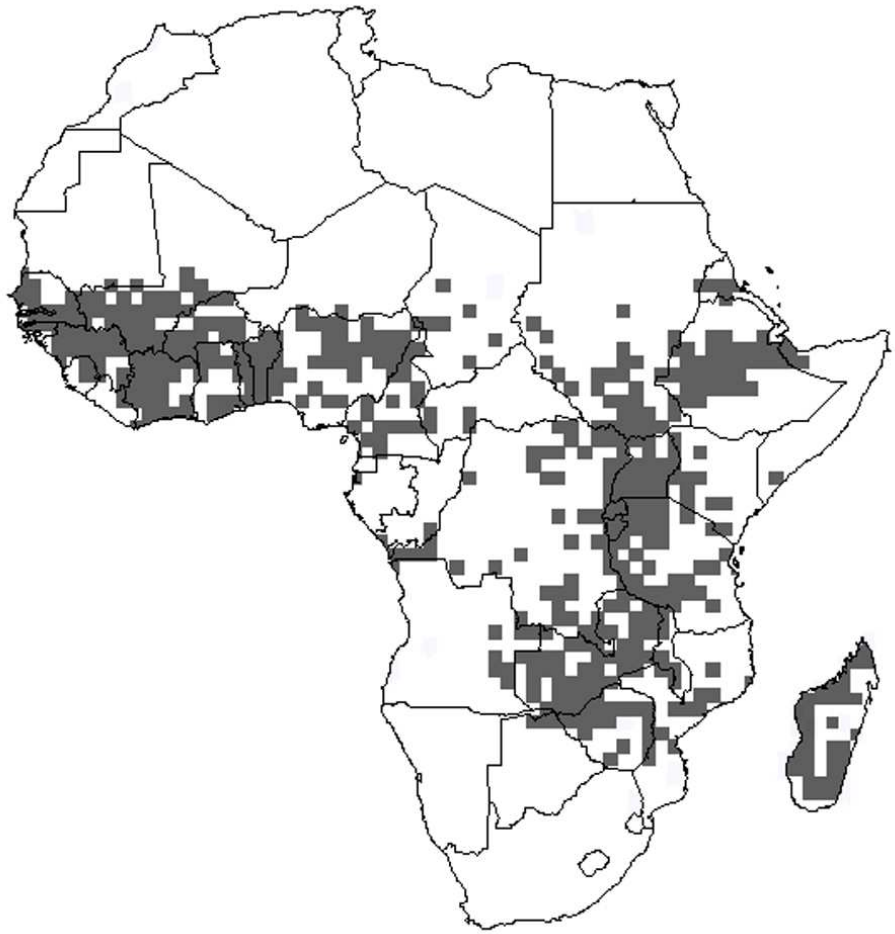
1.3 Heartwater or cowdriosis

1.3.1 History and distribution of the disease:

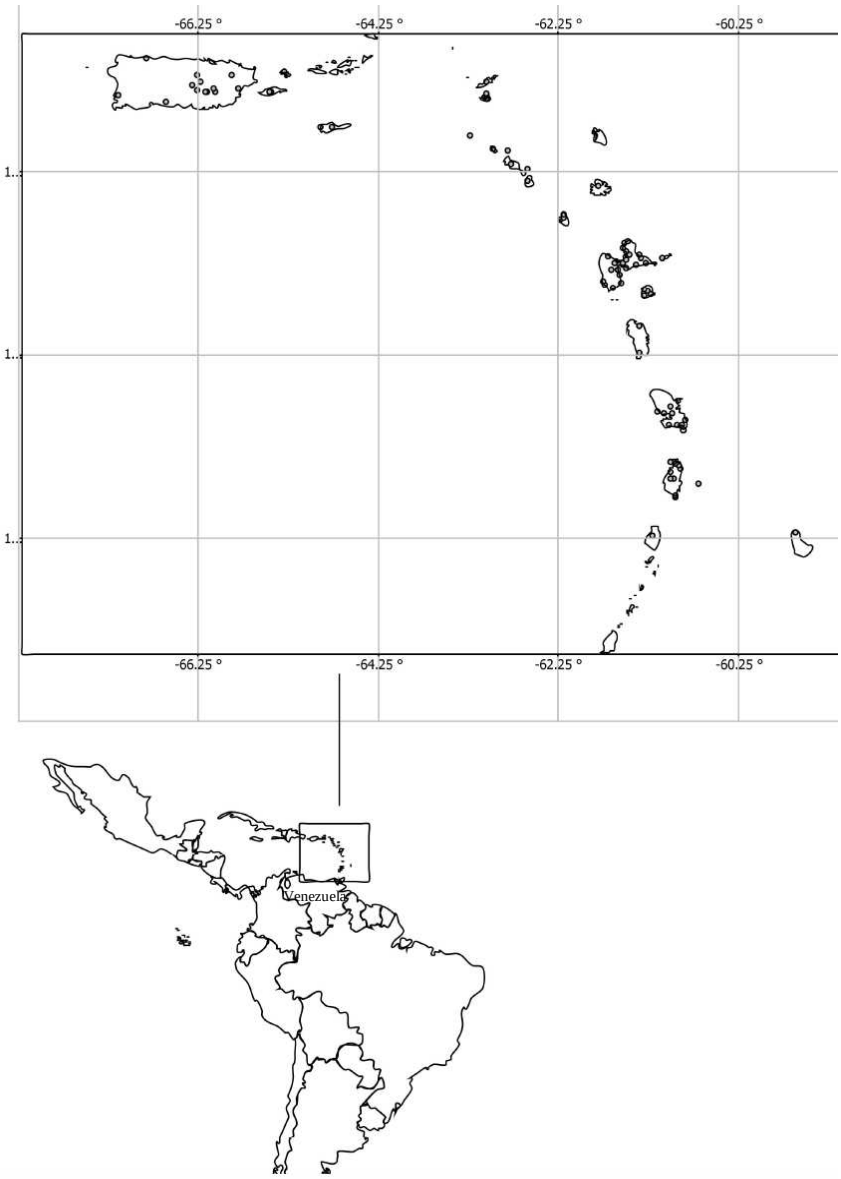
The first report of heartwater as a disease entity was recorded in 1877 in South Africa (reviewed in Camus et al., 1996). Early research carried out in South Africa demonstrated that the disease could be transmitted by the inoculation of infected blood into a susceptible animal and it was therefore concluded that the causative agent was microbial (Dixon, 1898; Hutcheon, 1900). *A. hebraeum* was shown to be a vector of the disease in 1900 (Lounsbury, 1900), and the pathogenic agent was identified as a rickettsial organism and isolated from tissues of affected animals and infected ticks by Cowdry in 1925 (1925a, b). From the early 20th century onwards, heartwater was reported in most other sub-Saharan African countries (Camus & Barré, 1982) and in several Caribbean islands (Uilenberg, 1983) and a number of other *Amblyomma* species were shown to be capable of transmitting the organism. The disease has not been reported from Asia, despite the presence of certain *Amblyomma* species (Camus et al., 1996). Nevertheless, all countries where known *Amblyomma* vectors are present are at risk of introduction of the disease, particularly where the disease is present in neighbouring countries. These countries include most of the Caribbean islands and the American continent, where *Amblyomma* species occur that have been shown capable of transmitting *E. ruminantium* experimentally. However, the establishment and spread of the disease is more likely to occur if the natural vector of heartwater, *A. variegatum* is also introduced (Barré et al., 1987). Fig. 1.4 shows the current distribution of *A. variegatum* in the African continent and Caribbean.

Fig. 1.4: Distribution of *A. variegatum* in Africa (A, squares) and in the Caribbean (B, circles). Maps were kindly provided by Prof. Agustin Estrada, Universidad de Zaragoza, Spain).

A



B



1.3.2 Clinical signs and pathogenesis:

Animals infected with *E. ruminantium* typically develop fever. The average incubation period to fever after natural infection is 18 days, and can vary depending on whether nymphs (15 days) or adults (21 days) transmit the disease. In experimentally infected animals, this incubation period is usually shorter than in natural infection but varies depending on the infecting dose, the source of the organisms (blood, tissue homogenate, ground-up tick suspension or infected ruminant endothelial cell culture), the virulence of the *E. ruminantium* isolate used and the mammalian host species (Camus et al., 1996). In general, small ruminants develop fever between 9 and 10 days following experimental infection with virulent strains of the organism.

Four forms of the disease have been described in small ruminants and cattle. Overall clinical signs are similar in the different ruminant species although nervous signs are less pronounced in cattle (Camus et al., 1996). In the peracute form, animals die suddenly without having shown obvious clinical signs. In the acute and most common form of the disease, a severe febrile reaction occurs and persists throughout the course of the disease; after one to two days of fever, anorexia, dyspnoea, a disturbed expression in the eyes and nervous signs appear (the animal is restless and moves in circles, shows sucking movements of the mouth and, when recumbent, galloping movements of the legs). The temperature shows an abrupt fall to sub-normal just prior to death. Recovery from this form of the disease is rare. In the subacute form, signs resemble those seen in acute cases but are less pronounced and a higher proportion of animals recover. The mild form is characterised by transient fever, which may not be noticed in the field, followed by recovery. This

form is of epidemiological importance as it allows development of immunity but results in carrier animals. Sheep, cattle, and African buffalo (*Syncerus caffer*) have been demonstrated as carrier animals (Andrew and Norval, 1989a; Bekker et al., 2002). Mortality rates vary between 5% and 100%, depending on the virulence of the *E. ruminantium* organisms, the infective dose and the breed of animal (Camus et al., 1996).

As indicated by the name "heartwater", hydropericardium is a striking feature at post-mortem examination of most animals that die of the disease. Hydrothorax, oedema of the lungs and splenomegaly are observed in the majority of the animals. Other regular findings are enlarged lymph nodes and congestion of the meninges with occasional meningeal oedema in the brain (Camus et al., 1996). Death may be due to extreme pulmonary oedema, cardiac insufficiency caused by pericardial distension, or as a result of circulatory collapse. The pathophysiological changes seem to centre on an increased capillary permeability that allows leakage of plasma proteins, resulting in transudation through the serous membrane. Cowdry (1926) reported that the presence of *E. ruminantium* causes deformation of the nucleus and distension of parasitised cells and suggested that this was responsible for the brain lesions and therefore for the nervous signs but not for the increased vascular permeability. It has been suggested that a toxin may causes the latter change (Camus et al., 1996) but such a toxin has never been demonstrated.

1.4 Microscopical findings

1.4.1 Morphological characteristics of *E. ruminantium*:

Cowdry in his original description of the morphology and staining characteristics of the organisms under light microscopy, reported that they stained negatively with Gram's stain, clear blue with the Giemsa method (and other basic aniline dyes) and red with the Fuchsin method (Cowdry, 1925a). He described the organisms as coccoid, 0.2-0.5 µm diameter, most often found in clumps (from ten to several hundred) enclosed in a vacuole situated to one side of the nucleus of the infected cell. Both the organisms and colonies were described as extremely pleomorphic. Apart from the cocci described by Cowdry, ring- and horseshoe-shaped forms, rods and bigger bulky irregular masses (up to 2-4 µm) can be seen. The morphology of the parasite is similar in the tick (Cowdry 1925b). Fig. 1.5 shows a Giemsa-stained brain smear containing typical colonies of *E. ruminantium* infecting endothelial cells of brain capillaries (A), and *E. ruminantium* in endothelial (B) and tick (C) cell cultures.

The ultrastructural morphology of *E. ruminantium* has been studied in endothelial cells in the choroid plexus of infected sheep (Piennar, 1970), in *Amblyomma* tick tissues (Kocan and Bezuidenhout, 1987; Kocan et al., 1987 a, b; Hart et al., 1991), *in vitro* in bovine endothelial cells (Prozesky et al., 1986; Prozesky and Du Plessis, 1987; Jongejan et al., 1991c) and in two different tick cell lines (Bell-Sakyi et al., 2000b). In all stages, *E. ruminantium* organisms are surrounded by two membranes,

and occur in colonies or morulae within membrane-bound vacuoles in the host cell cytoplasm. Based on the morphology of the internal structure, two types of organism are described: elementary bodies (electron-dense organisms) and reticulate bodies (larger and less dense). In endothelial cell cultures, a range of organisms between reticulate and electron-dense forms (intermediate organisms) can also be seen.

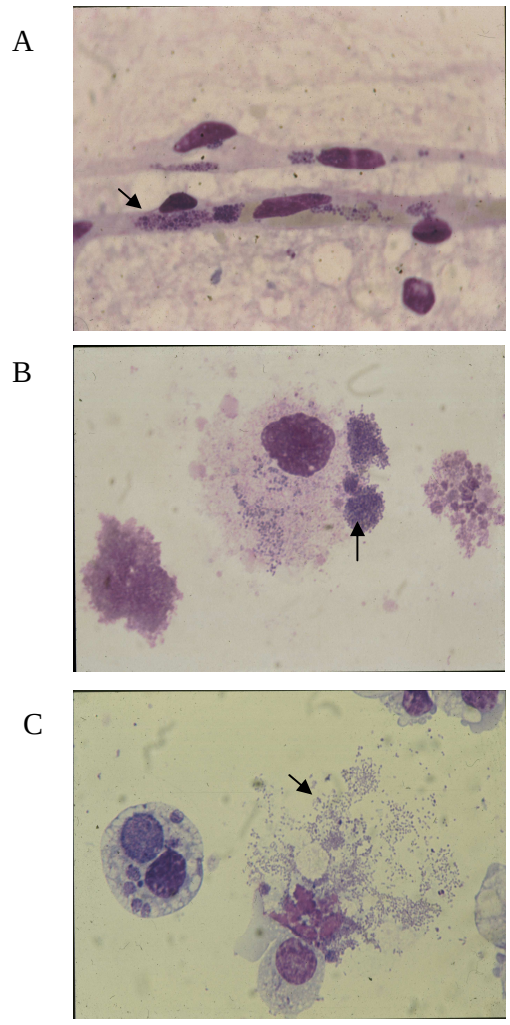


Fig. 1.5: Light micrographs of Giemsa-stained *E. ruminantium* in (A) brain crush smear of a goat infected with the Welgevonden isolate; (B) cytocentrifuge smear of Welgevonden-infected bovine endothelial cell cultures; and (C) cytocentrifuge smear of Gardel-infected IDE8 cell culture. Arrows are pointing to *E. ruminantium* colonies (A and B) and free bacteria (C). Magnification x 1000 oil immersion (These pictures were kindly provided by Dr. Lesley Bell-Sakyi).

1.4.2 Sites of replication in the mammalian and tick hosts:

Cowdry (1925a) reported that in experimentally infected animals, *E. ruminantium* organisms are detected within the endothelial cells of the smaller blood vessels of various organs including brain, lung, kidney, spleen and lymph nodes but were most easily observed in capillaries of renal glomeruli and the superficial grey matter of the cerebral cortex. The organisms have also been observed in cells of the reticulo-endothelial system (Du Plessis, 1975), macrophages (Du Plessis, 1970), monocytes (Pienaar, 1970) and neutrophils (Logan et al., 1987).

In the infected tick, the organism has been observed by light microscopy in the midgut epithelial cells and free in clumps in the lumen of intestines of moulting larvae (Cowdry, 1925b). Electron microscopy studies on infected nymphs and adult ticks have identified *E. ruminantium* colonies in midgut epithelial cells (Kocan & Bezuidenhout, 1987; Kocan et al., 1987b; Hart et al., 1991), salivary gland cells (Kocan et al., 1987a; Yunker et al., 1993), malpighian tubule cells (Bezuidenhout, 1988) and haemocytes (Hart et al., 1991). Inoculation into susceptible sheep of organ suspensions from infected ticks demonstrated that all organs tested, except the brain, haemolymph and ovaries, were infective (Bezuidenhout, 1988).

1.5 Diagnosis of heartwater

1.5.1 Microscopy:

None of the clinical signs observed in animals infected with *E. ruminantium* are pathognomonic for the disease. Definitive diagnosis of heartwater post mortem

depends on demonstration of *E. ruminantium* organisms in vascular endothelial cells, usually in capillary endothelial cells in stained smears prepared from brain tissue (Purchase, 1945).

1.5.2 Serology:

A number of serological tests have been used for diagnosis and assessment of the distribution of the disease. The first workable assay was based on the indirect fluorescent antibody (IFA) test using antigens produced *in vivo* in infected mouse macrophages (Du Plessis and Malan, 1987) or in caprine neutrophils (Logan et al., 1987; Jongejan et al., 1989). The advent of an *in vitro* culture system for the growth of *E. ruminantium* in endothelial cells (Bezuidenhout et al., 1985) permitted the production of large quantities of relatively pure antigen. An IFA test (Martinez et al., 1990; Asselbergs et al., 1993) and several enzyme-linked immunosorbent assays (ELISA), employing both indirect and competitive protocols, were developed based on purified, whole or sonicated endothelial cell-derived *E. ruminantium* elementary bodies or proteins solubilised therefrom (Jongejan et al., 1991b; Soldan et al., 1993; Martinez et al., 1993). These assays were of some value for large-scale epidemiological studies in most diagnostic laboratories but lacked specificity because of cross reactivity of antibodies induced by other *Ehrlichia* species. A competitive ELISA (Jongejan et al., 1991b) used a monoclonal antibody directed against an immunodominant 32 kDa (MAP1) protein located on the surface of *E. ruminantium* elementary bodies which was later found by immunoblotting to be conserved within the genus *Ehrlichia* (Jongejan et al., 1993; Mahan et al., 1993).

Having overcome the problem of source of antigen the question of specificity needed to be addressed. Other ehrlichial species that infect ruminants and therefore

potentially hinder the diagnosis of heartwater include *Anaplasma bovis, E. ovina, E. ondiri* and *Anaplasma phagocytophilum*. In order to reduce cross-reactivity, van Vliet et al. (1995) identified an immunogenic region of the MAP1 protein, the MAP1-B fragment, which did not cross-react with *A. bovis, E. ovina* or *A. phagocytophilum*. Although the ELISA developed with this antigen showed cross-reactions with *E. canis* and *E. chaffeensis*, these pathogens do not infect domestic livestock and therefore this cross-reactivity should not interfere with the use of the test for epidemiological studies of heartwater. The validation of the MAP1-B ELISA confirmed its usefulness for the detection of previous infection in small ruminants (Mboloi et al., 1999); however, with cattle the results have been less satisfactory as it was found that IgG antibody responses specific to MAP1-B and other *E. ruminantium* antigens are down-regulated in cattle despite repeated exposure to *E. ruminantium* via ticks (Semu et al., 2001). Recently, a polyclonal competitive ELISA (PC-ELISA) was described, which uses crude antigen from solubilised elementary bodies and polyclonal biotinylated competitor antibody for detection of antibodies to *E. ruminantium* (Sumption et al., 2003). Its use in the field (Bell-Sakyi et al., 2003) confirmed that this assay is as sensitive as the MAP-1B ELISA for the detection of *E. ruminantium* exposure in sheep and more sensitive than the MAP-1B ELISA to detect seroconversion in cattle. However, more validation needs to be done regarding the specificity of the test.

The main drawback of all serological tests in *E. ruminantium* diagnosis is that they only indicate previous exposure to the pathogen. Nevertheless, serology continues to be a useful tool for epidemiological surveys in heartwater-endemic areas.

1.5.3 Molecular detection:

A DNA probe, pCS20, was used to identify *E. ruminantium* in *A. variegatum* (Waghela et al., 1991) and *A. hebraeum* (Yunker et al., 1993) ticks. The probe hybridised to all eight heartwater isolates tested and was able to detect *E. ruminantium* DNA in plasma from infected sheep before and during the febrile reaction (Mahan et., 1992). The probe is highly specific for *E. ruminantium* and did not cross-react with *A. marginale*, *Rickettsia spp*., and *Neorickettsia* (previously *Ehrlichia*) *risticii*. A comparative evaluation of 16S ribosomal RNA, *map1* and pCS20 probes for the detection of *E. ruminantium* in ticks showed the pCS20 probe to be the most sensitive (Allsopp et al., 1998). Peter et al. (1995) showed that PCR amplification, using primers designed to amplify the pCS20 fragment, was more sensitive than DNA probe hybridisation in the detection of *E. ruminantium* in field ticks. The assay does not detect DNA of *E. chaffeensis*, *E. canis* or the novel white-tailed deer ehrlichia (WTDE) agent (Mahan et al., 2004). PCR assays, based on the *map1* and 16S ribosomal RNA genes, alone or combined with hybridisations, have been used for diagnosis of *E. ruminantium* in blood and bone marrow samples from clinically normal Zimbabwean ungulates (Kock et al., 1995), in blood samples collected from small ruminants in Mozambique (Bekker et al., 2002) and in *Amblyomma* ticks (Allsopp et al., 1998; Bekker et al., 2002). A nested PCR assay, based on the pCS20 fragment and the *map1* gene, has been developed and showed improvement of the sensitivity for each PCR target (pCS20 and *map1*) as compared to conventional PCR for detecting *E. ruminantium* in ticks or blood, brain and lung samples from ruminant hosts (Martinez et al., 2004). Additional advantages of PCR assays over tests involving hybridisation are the omission of the laborious

hybridization step and their suitability for automated high-throughput systems allowing analysis of large numbers of samples in epidemiological studies. However, careful assessment of this method needs to be done for its sensitivity in detecting low numbers of *E. ruminantium* in carrier animals.

Simbi et al. (2003) reported the pCS20 PCR assay to be more reliable than the MAP1-B ELISA in detecting exposure of cattle to *E. ruminantium* infection in a heartwater-endemic area; antibody responses to the MAP1-B antigen were negative while organisms were detectable by PCR. In the absence of a specific serological test, the use of probes and/or PCR becomes desirable for epidemiological studies.

1.6 Control measures

1.6.1 Treatment:

Control is usually achieved through treatment of clinical cases, tick control and/or vaccination. Tetracyclines constitute the drugs of choice for treatment. The use of antibiotics is only effective if applied early in the course of the clinical reaction; antibiotic treatment does not prevent the development of immunity and does not sterilise the animals from infection (Camus et al., 1996).

1.6.2 Tick control:

Tick control of heartwater involves application of acaricides to animals at risk of infection. The disadvantages of this method are that the acaricides need to be applied continuously and are therefore costly and time-consuming, the compounds

used are potentially hazardous to human health and the environment and, particularly if treatment is sustained over prolonged periods, it may result in development of acaricide resistance in tick populations. In addition, consideration of tick control must take into account the desirability of maintaining endemic stability. In endemically stable areas where tick control is not practised, a high level of immunity in stock, particularly cattle, is seen. In these areas, intensive tick control may actually increase losses due to heartwater, while selective control, e. g. acaricidal application only when the level of infestation causes tick-worry, tends to reinforce endemic stability and herds may develop a high degree of immunity. There is some doubt as to whether the concept of endemic stability is as valid for management of heartwater in small ruminants as it is in cattle (Camus et al., 1996; Yunker et al., 1996). In unstable or transitional areas, where intensive control has been practised, wildlife is excluded, or where *Amblyomma* are established but not heavily infected, intensive tick control or immunisation is recommended. The advantage of intensive tick control is that production losses due to tick-worry or tick-borne diseases are kept to a minimum. The main disadvantage is that it will prevent acquisition of immunity of the animals to tick-borne diseases due to lack of natural challenge and therefore the stock are vulnerable to disease if, for unforeseen reasons, acaricides become unavailable (Camus et al., 1996; Yunker et al., 1996).

1.6.3 Vaccination

1.6.3.1 Infection and treatment method:

Large-scale immunisation against heartwater has been carried out only with the infection and treatment method. This method consists of infecting animals with a

virulent preparation of *E. ruminantium* (eg. the Ball 3 isolate in sheep blood is used in South Africa) (Neitz and Alexander, 1945) and treating the infection as soon as the febrile reaction commences. The choice of vaccine stock is an important consideration due to significant antigenic and immunogenic differences among isolates (Du Plessis et al., 1989; Jongejan et al., 1991a). Although this method can protect animals, it is expensive and inconvenient because of the requirement of a cold chain for delivery, and it is unreliable as breakdowns in immunity are common and deaths associated with vaccination occur. As an alternative, exposing young animals to infected ticks by introducing them into endemically stable areas during times of tick activity, rather than vaccination, is recommended to confer long-term immunity (Camus et al., 1996).

1.6.3.2 Attenuated vaccines:

A Senegalese stock of *E. ruminantium* was found to be attenuated for virulence in small ruminants after only eleven passages (seven months in culture) in bovine umbilical endothelial cells, while fully retaining its immunogenicity (Jongejan, 1991). The attenuated culture stock was able to protect goats and sheep against virulent homologous challenge with a blood stabilate, but not against challenge from *E. ruminantium* isolates from geographically diverse backgrounds (Gueye et al., 1994). In contrast, the South African Welgevonden isolate, attenuated after more than 50 passages in a canine macrophage-monocyte cell line (DH82) and re-adapted to grow in a bovine endothelial cell line (BA 886), protected sheep against a lethal needle challenge with the virulent homologous stock or with one of four different heterologous stocks (Ball3, Gardel, Mara 87/7 and Blaauwkrans)

(Zweygarth et al., 2005). The Welgevonden attenuated vaccine, since it is produced in a closed *in vitro* culture system, could be cheaper and safer than the blood vaccine, and the expected range of cross-protection appears to be wider than with the Ball 3 blood vaccine. A single dose without treatment seems to be sufficient to induce immunity. However, the efficiency of the vaccine has yet to be fully assessed against tick challenge in the field and the possibility that the attenuated organisms might revert to virulence after repeated passage through ticks in the field needs to be investigated.

1.6.3.3 Inactivated vaccines:

An inactivated vaccine consisting of culture-derived *E. ruminantium* organisms that are chemically inactivated and combined with an adjuvant has been developed. Successful immunisation against homologous challenge has been reported in goats (Martinez et al., 1994), sheep (Mahan et al., 1995) and cattle (Totté et al., 1997). This vaccine (using the Mbizi isolate from Zimbabwe) has also been shown to protect sheep against heterologous strains and against laboratory and field tick challenge (Mahan et al., 1998). Although much safer than live vaccines, this inactivated vaccine required several doses spread over a period of weeks or months, during which the animals had to be kept tick-free. While vaccination resulted in reduction in mortality, it had little effect on morbidity (Martinez et al., 1996; Mahan et al., 2001). This vaccine has the advantage that it can be easily modified to include any isolate of *E. ruminantium* which can be cultivated *in vitro*. Optimization of a cost-effective process for the mass production of the inactivated *E. ruminantium* vaccine is in progress (Marcelino et al., 2005, 2006). Studies of the immune

responses elicited by this protocol of vaccination have demonstrated a $CD4^+$ T-cell response against *E. ruminantium* antigens, including the major antigenic proteins MAP1 and MAP2, and production of IFN-γ (Totté et al., 1997). *E. ruminantium*-specific T-cell lines generated in immunised animals have been used to identify antigens that might be important in inducing protective responses and hence be of potential value for the development of improved subunit vaccines.

1.6.3.4 DNA vaccines:

An experimental DNA vaccine incorporating a gene encoding the immunodominant MAP1 protein of *E. ruminantium* (Crystal Springs strain) was shown to confer protection to 23-88% DBA/2 mice against a homologous challenge, which killed 143 out of 144 control mice (Nyika et al., 1998). Boosting of the DNA vaccine-primed mice with recombinant MAP1 protein increased the level of protection against death from homologous challenge, although protection was still incomplete (53-67%) (Nyika et al., 2002). Immunised mice mounted a T helper cell (Th1-type) response, characterised by production of IFN-γ and IL-2 in supernatants of splenocyte cultures stimulated with *E. ruminantium* lysates or recombinant MAP1 antigen. Antibodies generated against MAP1 were predominantly of the IgG2a isotype, characteristic of a Th1 type immune response. Although encouraging, the findings could not be reproduced in cattle (van Heerden et al., 2004). Another attempt at immunisation with DNA utilised four *E. ruminantium* (Welgevonden isolate) genes (2, 3, 5 and 6) from a genetic locus involved in nutrient transport. Immunisation of sheep with an equimolar cocktail of four plasmids, each containing one gene cloned in separate pCMViUBs DNA vaccine vectors, engendered 100%

protection against subsequent lethal needle challenge with the homologous isolate and with each of five different virulent heterologous isolates (Ball3, Blaauwkrans, Gardel, Kwanyanga, and Mara 87/7). However, when sheep immunised with this cocktail were exposed to tick challenge in a heartwater-endemic area, only a few animals survived (Collins et al., 2003).

Vaccination is the most useful disease control method for introducing improved or imported animals into heartwater-endemic areas, unless all transmission can be prevented by tick control. Morever, where resistance of *Amblyomma* to acaricides exists, vaccination may be the only possible method of control. Further research is required into the development of safe and effective methods of vaccination against heartwater.

1.7 Antigenic and molecular characterisation of *E. ruminantium*

1.7.1 MAP1 protein:

An immunodominant response to a protein of approximately 32kDa was observed in sera from recovered goats, sheep and cattle (Jongejan & Thielemans 1989, Rossouw et al., 1990). *E. ruminantium* antigens were obtained from Senegal- or Welgevonden-infected choroid plexus and larger brain blood vessels of goats. Polyvalent sera raised in goats and mice, infected with one each of nine different and geographically separated isolates of *E. ruminantium*, were found to recognise a protein of approximately 32 kDa. This protein, conserved among *E. ruminantium*

isolates, was designed Cr32 (Jongejan & Thielemans, 1989). Differences in molecular weight, depending on the origin of the *E. ruminantium* stock, led to the renaming of the protein as major antigenic protein 1 (MAP1) (Barbet et al., 1994). Jongejan et al. (1991b) raised five monoclonal antibodies against bovine endothelial cell culture supernatant containing elementary bodies of the Welgevonden isolate. Four of these monoclonal antibodies recognised a 32kDa protein (MAP1) in Western blots containing three different stocks of *E. ruminantium*. Electron microscopy and immunogold labelling of *E. ruminantium* organisms *in vitro* using the monoclonal antibody 4F10B4 demonstrated that MAP1 is on the surface of elementary bodies (Jongejan et al., 1991b). MAP1 is conserved within the genus *Ehrlichia* (Jongejan et al., 1993). Three other less immunodominant antigens of 21 kDa (MAP2) (Mahan et al., 1994; Bowie et al., 1999); 27 kDa (Rossouw et al., 1990) and 43 kDa (Shompole et al., 2000) have also been identified.

1.7.2 Multigene families in the family *Anaplasmataceae*:

A multigene family is defined as a cluster of related genes with identical or similar nucleotide sequences, encoding related proteins. The classic model for evolution of a multigene family involves gene duplication followed by sequence divergence of the duplicated genes.

Several studies have identified the presence of homologous multigene families in tick-transmitted pathogens of the family *Anaplasmataceae* such as *E. ruminantium (map1), E. chaffeensis (omp1), E. canis (p30), A. phagocytophilum (p44)* and *A. marginale (msp-2)* (Sulsona et al., 1999; Ohashi et al., 1998a; Ohashi et al., 1998b; Palmer et al., 1994; Zhi et al., 1999). The recent complete annotation of the genome

sequences of *E. ruminantium* (Collins et al., 2005) and related organisms (Brayton et al., 2005; Hotopp et al., 2006) has resulted in detailed information on the extent of the gene families and the mechanisms by which they are regulated. These reports have found that *Ehrlichia* and *Anaplasma* have undergone variable levels of expansion within their respective genomes. The genome of *A. phagocytophilum* carries the *P44* multigene family in addition to *msp2* and *omp-1* gene families, whereas *A. marginale* has the *msp2* and *omp-1* families but lacks the *P44* genes. *Ehrlichia* spp. (*E. ruminantium*, *E. chaffeensis*, *E. canis*, *Ehrlichia ewingii* and *Ehrlichia muris*) have only an *omp-1* multigene family (Lin et al., 2004). These findings suggest that *msp2*, *P44* and *omp-1* genes arose and co-evolved within a common ancestral genome by gene duplication. The significant sequence identity between members of the different gene families (*omp-1/msp-2*/*p44)* supports the hypothesis that these gene families originated from a common ancestor and duplicated prior to speciation (Yu et al., 2000). For instance, the protein sequence identities between MAP1, MAP1-1, and MAP1-2 in *E. ruminantium* (Senegal) are lower (32.8-46.7%) when compared to each other than to orthologs in *E. canis* and *E. chaffeensis* (Bekker et al., 2002). The highest percentage of identity of MAP1-2 is found with *E. canis* P28-1 (57.4%) and *E. chaffeensis* P28-11 (56.5%), MAP1-1 with *E. canis* P30-10 (79.7%) and *E. chaffeensis* OMP-1B (78.0%) and MAP1 with *E. canis* P30 (56.5%) and *E. chaffeensis* P28 (67.2%). Based on this model, it is suggested that *A. marginale* duplicated *msp2* genes but may have lost its *p44* genes while *Ehrlichia spp*. duplicated *omp-1* genes extensively and lost both *p44* and *msp2* genes. On the other hand, *A. phagocytophilum* duplicated the *p44* genes extensively (~100 copies) while the *msp2* polycistronic expression locus in *A. marginale*

underwent a process of degeneration (currently less than 10 copies) (Lin et al., 2004; Brayton et al., 2005; Hotopp et al., 2006). It has been speculated that duplications of particular groups of genes encoding major outer membrane proteins may have facilitated their adaptation in different host cell types (erythrocytes, granulocytes, monocytes and endothelial cells), in different mammal host species, and in different tick species (Lin et al., 2004). Fig. 1.6 shows a phylogenetic tree of OMP1/MSP-2/P44 proteins.

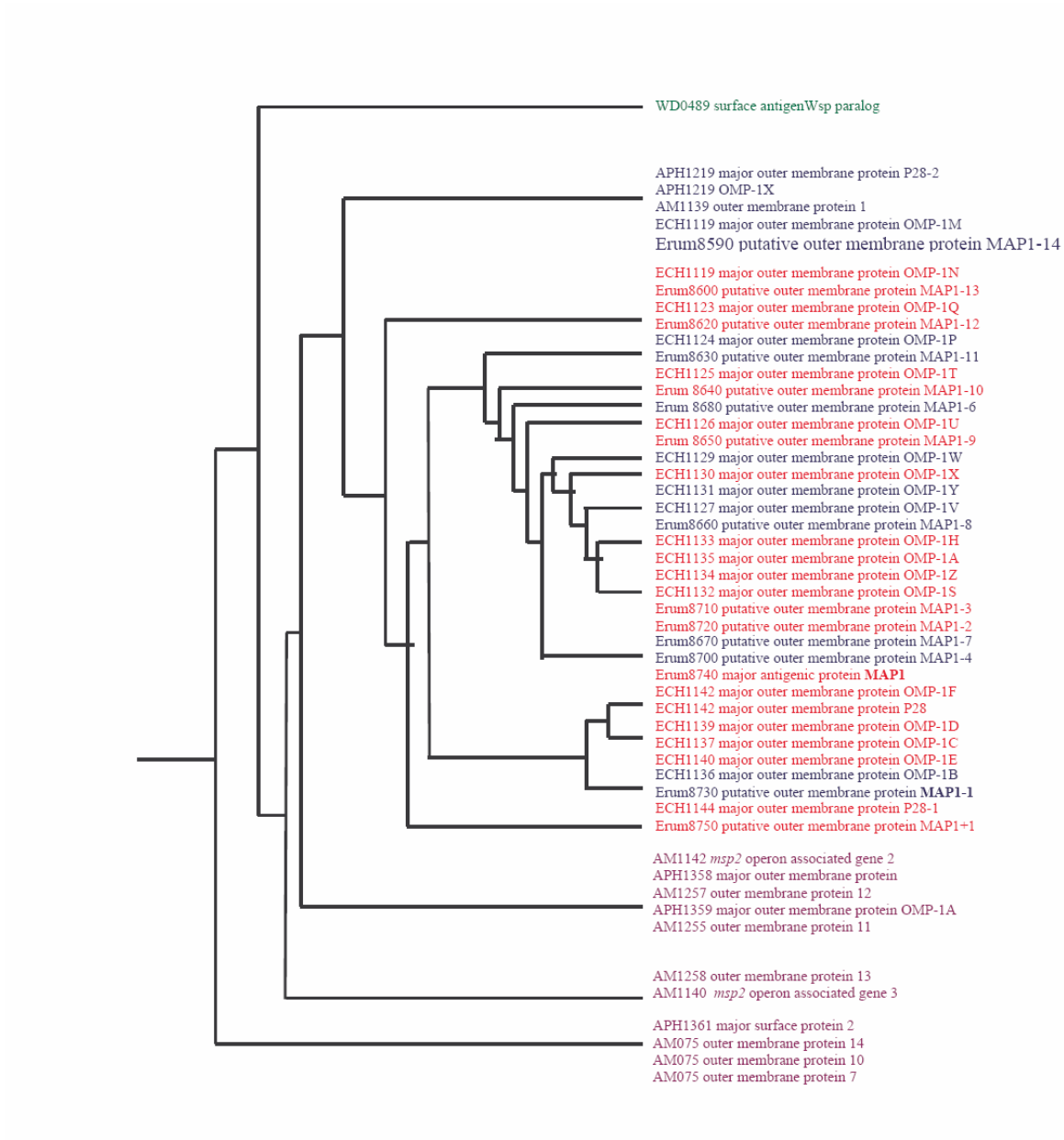


Fig. 1.6: Phylogenetic tree inferred from alignment of sequences of outer membrane protein superfamilies including *E. ruminantium*, *E. canis*, *A. phagocytophilum* and *A. marginale.* This arrangement shows that some MAP1 proteins are closer to e.g. *E. chaffeensis* OMP proteins than to those within the MAP1 superfamily (Data obtained from Hotopp et al., 2006, supplementary material, figure S2)

1.7.3 The *map1* multigene family:

The complete 1,516,355 bp sequence of *E. ruminantium* (Welgevonden strain) allowed identification of several families of genes encoding hypothetical membrane proteins; the major antigenic protein 1 (*map1*) gene family, for which orthologs are present in *E. canis*, *E. chaffeensis*, *A. phagocytophilum* and *A. marginale*; and two other potentially interesting membrane protein families, of 14 and 10 members, neither of which has orthologs in other sequenced genomes (Collins et al., 2005).

The 24,993 bp contig containing the *map1* multigene family has 21 ORFs. Sixteen of the 21 have homologies to *map1* (including *map1* itself) and 15 of them, from *map1* upstream to *map1-14*, were tandemly organised in a head-to-tail arrangement. The ORF downstream of *map1* (*map1+1*), is on the opposite strand. At the 5' end of the locus there is an ORF with 73.6% identity to the hypothetical transcriptional regulator (*tr*) of *E. chaffeensis*, and at the 3' end, an incomplete ORF showed 48.3% identity to the *secA* gene of *Rickettsia prowazekii*. Three ORFs containing sequences unrelated to *map1* paralogs were identified in the locus; *unknown* (*un) 1* is located between *map1-13* and *map1-12* while *un2* and *un3* are located downstream from *map1+1*. Thirteen paralogs at the 5' end of the locus were connected with short intergenic regions (ranging from 0 to 42 bp) and the remaining 3 paralogs at the 3' end were connected by longer intergenic regions (ranging from 375 to 1612 bp) (Fig.1.7).

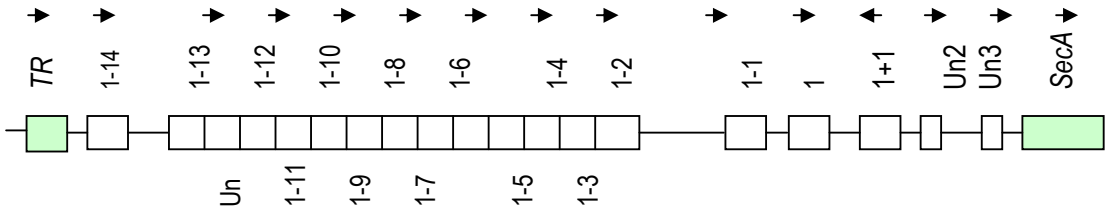


Fig. 1.7: Schematic representation of the *E. ruminantium map1* multigene family. Genes are represented by open boxes with arrows indicating their orientation in the cluster. *TR*: 73.6% identity to hypothetical transcription regulator gene of *E. chaffeensis. Un*: Genes with unknown function, between 10.5 and 35.2% identity to unknown function genes in *E. canis* and *E. chaffeensis*. *SecA:* 48.3% identical to the preprotein translocase *secA* subunit of *R. prowazekii.* (Data taken from van Heerden et al., 2004).

The protein identities among *map1* paralogs range from 13.3% to 66.5%, with a mean of 35.1%, for the Welgevonden isolate (Collins et al., 2005).

The 16 *map1* paralogs are predicted to encode proteins of between 24.3 and 35.6 kDa, with isoelectric points ranging between 5.74 and 9.96 (van Herdeen et al., 2004). The number of paralogs and the gene order in the *map1* cluster are highly conserved between *E. ruminantium* strains, as reported by Frutos et al. (2006) and Bekker et al. (2005) based on comparison of the *map1* locus sequences of the Welgevonden, Senegal and Gardel isolates. However, *E. ruminantium* seems to be capable of undergoing genomic rearrangements. Comparison of two subpopulations of the Gardel isolate maintained in different laboratories demonstrated that recombination between two genes (*map1-3* and *map1-2*) had occurred with the consequent deletion of *map1-2* in one subpopulation (Bekker et al., 2005).

Comparative genomic analysis revealed the cluster of *map1* paralogs as a group of genes potentially involved in the observed experimental host range differences, since the corresponding paralogs display a large number of substitutions and insertion/deletions between the two isolates Welgevonden, which is infective and pathogenic to mice, and Gardel, which is not. However, the *map1-1* gene, which has been shown to be preferentially expressed in *E. ruminantium*-infected tick cell cultures and in ticks (Bekker et al. 2002, 2005) is identical between the *E. ruminantium* Gardel and Welgevonden isolates (Frutos et al., 2006) suggesting that it is involved in functions that require strong protein sequence conservation. Several *map1* paralogs, such as *map1-2* and *map1-6*, show a large number of mutations between Gardel and Welgevonden isolates, but their synonymous/nonsynonymous (S/NS) substitution rates are not sufficiently different to conclude that functional

pressure is acting. Only *map1* and *map1-13* genes were biased toward synonymous (silent) substitutions, suggesting that protein function is conserved (Frutos et al., 2006). This is despite the fact that there is a strong immunodominant response to MAP1 in infected ruminants.

1.7.4 Transcriptional studies of the *map1* multigene family:

All 16 paralogs were found to be transcribed when *E. ruminantium* (Senegal, Gardel or Welgevonden isolates) were cultured in a range of different bovine endothelial cells at 30º and 37º C (van Herdeen et al., 2004; Bekker et al., 2005). Paralogs with short intergenic regions were co-transcribed with their adjacent genes, while *map1-13*, unknown (*un*) and paralogs downstream from *map1-2* were monocystronically transcribed (van Herdeen et al., 2004). In contrast, between 4 and 11 paralogs were found to be transcribed in different tick cell lines infected with Gardel or Welgevonden isolates. For all tick cell lines tested, a transcript for *map1-1* was present and predominated. Co-transcription of *map1* paralogs has not been studied in tick cell cultures.

1.7.5 Comparison of the *E. ruminantium map1* family with multigene families in related pathogens:

There is synteny between the upstream region of the *map1* gene family and its counterparts in other *Ehrlichia* species and the corresponding regions in the *msp-2/p44* multigene families, represented by the operon regions, *tr-omp1-opag3-opag2-opag1-msp2* in *A. marginale* (Löhr et al., 2004), and *tr-omp1X-omp1N-p44-msp-2* in *A. phagocytophilum* (Barbet et al., 2005), in both arrangement and the predominant polycistronic transcription pattern (Fig. 1.8). However, based on sequence similarity

and gene organization of the whole cluster, the *map1* multigene family is more closely related to the *omp1* and *p30* in *E. chaffeensis* and *E. canis* respectively, than to the *msp-2* and *p44* multigene families in *Anaplasma*.

E. chaffeensis, *E. canis*, and *E. ruminantium* have 16-25 tandemly repeated full-length genes arranged in one (*E. ruminantium* and *E. chaffeensis*) or two clusters (*E. canis*), flanked by a *tr* and a preprotein translocase (*SecA*) (Fig.1.9). These genes all have signal peptides and are likely to be secreted across the cytoplasmic membrane by *SecA* (Yu et al., 2000; Ohashi et al., 2001; van Heerden et al., 2004). Experimental studies have demonstrated that the gene *p28-19* of *E. chaffeensis* and the *map1* gene of *E. ruminantium* encode surface-exposed proteins (Jongejan et al., 1991b; Ohashi et al., 1998a).

Dot plot comparisons of the *map1* multigene locus with itself, and with the *omp* clusters of *E. chaffeensis* and *E. canis*, showed that there are three regions (α, β, and γ) containing repetitive elements interspersed with regions with non-repetitive elements in the locus (Ohashi et al., 2001; van Heerden et al., 2004). Repetitive elements are expected to be involved in genome fluidity and sometimes antigenic variation (Parkhill et al., 2000). The α region contains only the *map1* gene in *E. ruminantium* but five and six paralogs in the same region in *E. chaffeensis* and *E. canis* respectively. The percentage identities between *map1* and the genes in the α region of *E. canis* and *E. chaffeensis* ranged from 48.5% to 63.1%. The β region in *E. ruminantium* consists of *map1-2* and *map1-3* genes and the γ region comprises five genes from *map1-10* to *map1-6*. The non-repetitive regions consisted of the area upstream of *map1-11*, the region from *map1-5* to *map1-4*, *map1-1* and *map1+1* (Fig 1.9).

Map1, omp1 and *p30* paralogs are regulated by mechanisms of both monocystronic and polycistronic transcription, whereas transcription of *msp-2/p44* paralogs, which are dispersed throughout the genomes of *Anaplasma* spp., is more complicated. All 22 paralogs in the *E. canis p30* multigene family, and 16 paralogs in the *E. chaffeensis omp1* multigene family were found to be transcribed in infected monocyte cultures (Ohashi et al., 2001; Long et al., 2002). The *p30* paralogs with short intergenic regions were co-transcribed (Ohashi et al., 2001). These findings are similar to those observed in the *map1* multigene family of *E. ruminantium*, where all 16 paralogs were found to be active, mono- or polycistronically transcribed, in infected bovine endothelial cell cultures (this chapter, section 1.7.4). *In vivo* in infected vertebrates, 16/22 and 11/14 paralogs studied were found to be transcribed for *E. chaffeensis* and *E. canis* respectively. Interestingly, only 1 paralog, the *omp1B* and *p30-10* of *E. chaffeensis* and *E. canis* respectively, was found to be transcribed in various tick stages (Unver et al., 2001; Unver et al., 2002; Felek et al., 2003). *Omp1-B* and *p30-10* are orthologs of the *map1-1* gene in *E. ruminantium*. Bekker et al. (2002) reported transcription of the *map1-1* gene in *A. variegatum* ticks.

Alignment of predicted amino acid sequences of the *E. chaffeensis* OMP1 proteins, along with that of *E. ruminantium* MAP1 protein, revealed that the protein coding sequences of the genes have four long stretches of conserved regions separated by three hypervariable regions, where substitutions or deletions of amino acids were found (Ohashi et al., 1998a). Essentially, there is no difference in the positions where the constant and variable regions are located between *E. chaffeensis*, *E. canis* and *E. ruminantium* (Reddy et al., 1998). These hypervariable regions are present in all members of the *omp-1* of *E. chaffeensis*, however, clear conserved and

hypervariable domains such as those found in MAP1, were not detected in MAP1-1 or MAP1-2 indicating that the *map1* multigene family of *E. ruminantium* contains both conserved and variable genes (Bekker et al., 2002). The *map1* gene is highly polymorphic between different isolates of *E. ruminantium* (Reddy et al., 1998; Allsopp et al., 2001) while the *map1-1* gene is highly conserved (Sulsona et al., 1999).

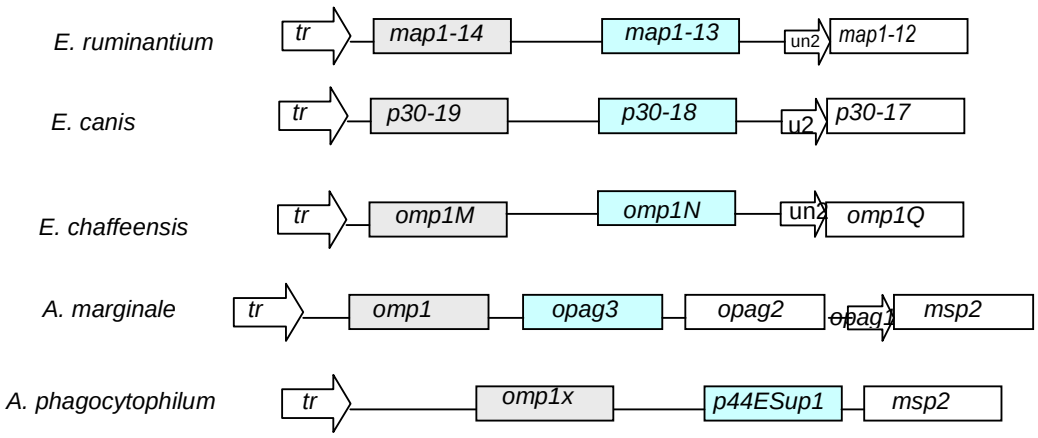


Fig. 1.8: Schematic representation of the upstream region of the *map1*, *omp1* and *p30* gene families of *E. ruminantium, E. chaffeensis* and *E. canis* respectively, compared to the corresponding regions in the *msp-2/p44* multigene families, represented by the operon regions in *A. marginale*, and *A. phagocytophilum* (Data was taken from Löhr et al., 2004, Barbet et al., 2005 and van Heerden et al., 2004).

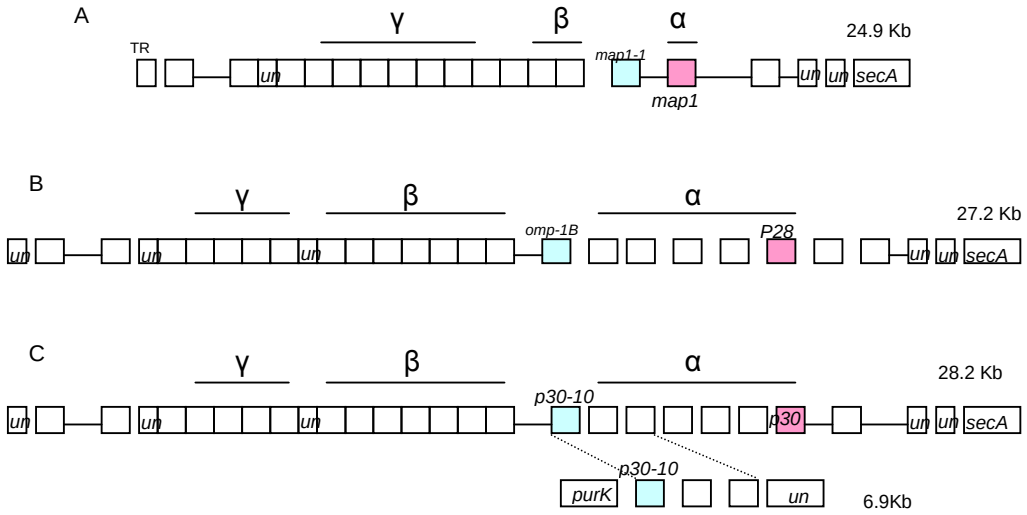


Fig. 1.9: Schematic representation of gene organisation of the *map1* (A), *omp1* (B) and *p30* (C) multigene families of *E. ruminantium, E. chaffeensis and E. canis* respectively. Genes are represented as open boxes and genes orthologous to *map1* and *map1*-1 are coloured. Symbols α, β and γ represent repetitive regions (Data was taken from Ohashi et al., 2001 and van Heerden et al., 2004).

1.7.6 Potential function of the *map1* multigene family in comparison to multigene families in related pathogens:

Since there is no epidemiologically significant transovarial transmission of pathogens of the family Anaplasmataceae in ticks, despite reports which apparently show that such transmission can occasionally occur (Bezuidenhout & Jacobzs, 1986; Shimada et al., 2004), ticks must acquire Ehrlichia or Anaplasma by feeding on an infected vertebrate. There is evidence from studies of Anaplasma species that multigene families encoding outer membrane proteins provide a system for generating antigenic variation, allowing persistence in the vertebrate host which thus becomes a reservoir of infection and facilitates effective tick transmission.

In *A. marginale,* MSP-2 is composed of conserved, amino- and carboxi-terminal regions flanking a single central hypervariable region (French et al., 1998). The multigene family encoding the MSP-2 consists of an expression site (*tr-omp1-opag3-opag2-opag1-msp2*) (Löhr et al., 2004), polycistronically transcribed, and 7 to 10 *msp2* gene copies (pseudogenes), dispersed over the genome, that lack part of the 5' and 3' conserved regions but do have the whole hypervariable region (Palmer et al., 1994). This multigene family is regulated by extensive intragenic recombination of either a whole hypervariable region (gene conversion) or a single segment, between the expression site and other *msp2* copies, employing flanking conserved regions, which result in formation of different sequences in the *msp2* hypervariable region and thus in antigenic variation (Barbet et al., 2001, French et al., 1999, Barbet et al., 2000, Brayton et al., 2001, Brayton et al., 2002, Futse et al., 2005). This mechanism explains what is seen in the infected host. In cattle, the MSP-2 variants observed in acute infection are cleared and replaced by newly expressed MSP-2

variants during early persistent rickettsemia, and again these are replaced by a new set of MSP-2 variants expressed at a second time point during persistent infection (Rurangirwa et al., 1999).

Brayton et al. (2005) reported recently that the surface coat of the *A. marginale* pathogen is dominated by two families containing immunodominant proteins: the *msp2* superfamily and the *msp1* superfamily. MSP1 is a surface exposed heteromeric complex consisting of MSP1a and MSP1b, which have been proved to be adhesins for host cells (Blouin et al., 2003). The *msp2* superfamily is built around *msp2*, *msp3* and *msp4*, the latter two molecules having a low level of sequence identity to *msp2*. MSP2, -3 and -4 reside in the outer membrane protein with surface exposed domains, with MSP2 and -3 being immunodominant proteins. Additional reports have shown that *A. marginale* uses extensively various multigene families to antigenically vary important, immunodominant surface antigens (Brayton et al., 2003).

In *Ehrlichia* spp., multigene families are not regulated by gene conversion of pseudogenes into an expression site, but by mechanisms of both monocystronic and polycistronic transcription of their paralogs. *Omp-1* genes of *E. chaffeensis* encode variable domains and unique epitopes that are recognised by antibodies (Zhang et al., 2004a); however, they do not seem to play a role in immune evasion. Sera from experimentally *E. chaffeensis*-infected dogs were evaluated for the presence of specific antibodies to OMPs by enzyme-linked immunosorbent assay as an indirect means of monitoring protein expression. Antibodies specific to all *omp1* proteins were detected during the infection period and the peak response to all the peptides appeared simultaneously in each dog. Concurrent expression of all OMPs in

persistently infected dogs does not support the hypothesis that sequential expression of the OMPs is important for persistent infection.

Findings such as variability of MAP1 amongst different *E. ruminantium* isolates and presence of a *map1* locus with all paralogs transcriptionally active indicate that genes in these regions could be involved in mechanisms to evade the immune response in the mammalian host, especially in carrier animals with long-standing persistent infections. Nevertheless, the lack of support for positive selection pressure on *map1*, despite the fact that MAP1 is serologically immunodominant, indicates that this protein is not important in allowing the parasite to evade the host immune response (Allsopp et al., 2001). It has been suggested that the function of the MAP1 protein is to divert the immune response away from paralogs that are important to the survival of the organism (van Herdeen et al., 2004). Differential transcription of *map1* genes between tick and bovine endothelial cell cultures, along with the single transcription of *p30/omp1* genes respectively in *E. canis* and *E. chaffeensis* infected ticks, may indicate that these multigene families confer to ehrlichial pathogens antigenic environmental adaptation, essential during tick transmission. The exact biological functions of the proteins encoded by ehrlichial gene families remain to be elucidated.

1.8 Aims of the present study

Succesful bacterial pathogens have evolved a variety of specific gene products that facilitate their survival and growth within the vector and the host, as well as mechanisms to regulate expression of these virulence-associated genes in response to their environment. For instance, the spirochaete *Borrelia burgdorferi* changes its outer surface during its alternating infections in ticks and mammals (Schwan and Piesman, 2002). OspA is abundantly expressed in *B. burgdorferi* in the guts of unfed ticks. As the tick starts to feed, most spirochaetes cease to express Osp A on their surface and start to express a different Osp, Osp C. This change in protein expression is correlated with the exit of spirochaetes from the midgut, dissemination through the haemolymph and passage to the salivary glands of feeding ticks and transmission to the host. Therefore the detection of genes that are differentially transcribed under different conditions has become a central aspect of research on pathogenesis of bacterial diseases.

Previous studies using systems for propagation of *E. ruminantium in vitro* in mammalian and tick culture systems, have demonstrated clear differences in morphology of organisms grown in endothelial and tick cell cultures (Bell-Sakyi et al., 2000a, b) and also differences in their immunogenicity and pathogenicity for sheep. Bell-Sakyi et al. (2002) reported that most sheep (98.33%) inoculated intravenously with tick cell cultures heavily infected with *E. ruminantium* showed no clinical response and were, some of them (~50%), even protected against homologous challenge with *E. ruminantium*-infected endothelial cell cultures, which

in contrast induced severe heartwater in all the control sheep. Such differences are likely to reflect host-dependent differences in bacterial protein expression. Given the evidence of differential transcription of multigene families encoding outer membrane proteins in vertebrate and tick hosts, observed in related ehrlichial agents, the *map 1* family of genes of *E. ruminantium* were considered to represent candidates likely to be involved in determining these differential biological properties.

The main purpose of the studies described in this thesis was to investigate the differential expresion of genes of *E. ruminantium*, with emphasis on those of the *map 1* multigene family, during different stages of development of the organisms in their life cycle, in order to provide insight into the potential role of gene family members in the pathogen's adaptation to the vector and the mammalian host.

1.8.1 Objectives

a) To determine the kinetics of *E. ruminantium* in the mammalian host infected with *E. ruminantium*-derived endothelial cells, by quantification of bacteria in peripheral blood using real-time PCR.

b) To determine by RT-PCR whether the pattern of transcription of the *map1* multigene family of *E. ruminantium* grown in tick cells at 31º C (tick vector-like temperature) differs from that in *E. ruminantium* grown in tick cells at 37º C (mammalian temperature).

c) To determine if the pattern of transcription of the *map1* multigene family in *E. ruminantium* elementary bodies, the forms infective for mammals, is different to that transcribed by stages in tick cells, non-infective for the mammalian host, and assess whether such differences are correlated with infectivity.

d) To identify genes of the *map1* multigene family of *E. ruminantium* which may be differentially transcribed during the different developmental stages of the bacteria in different tissues of infected *A. variegatum* ticks.

e) To identify host cell-specific *E. ruminantium* proteins, encoded by the *map1* cluster, utilising organisms grown *in vitro* in either tick or bovine endothelial cell cultures.

f) To identify genes, other than the *map1* cluster, differentially expressed by *E. ruminantium* in tick and endothelial cells, by suppression subtraction hybridisation (SSH).

Chapter 2: Development of a real-time PCR for quantitation and kinetics of experimental *E. ruminantium* infection of sheep

2.1 Introduction

Animals have been immunised experimentally with *E. ruminantium* using attenuated (Jongejan, 1991; Zweygarth et al., 2005), inactivated (Martinez et al., 1994; Mahan et al., 1995; Esteves et al., 2004) and virulent organisms grown in endothelial (Bekker et al., 2002) or tick cells (Bell-Sakyi et al., 2002, Bell-Sakyi, 2004). Inoculation of sheep with *E. ruminantium* infected blood (blood stabilates) or cultivated in mammalian endothelial cell cultures is almost always followed by a severe clinical reaction, and animals recovered after treatment seroconvert and are protected against subsequent challenge with virulent stabilates (Bezuidenhout, 1987a). However, the sequence of events leading to establishment of infection with *E. ruminantium* in the mammalian host and the kinetics of appearance of organisms in the circulation of infected animals is poorly understood.

The aim of the work described in this chapter was to investigate the kinetics of infection induced by *E. ruminantium* derived from mammalian cell cultures or infected blood by quantification of organisms in the peripheral blood of infected sheep.

Methods used previously to quantify *in vitro* culture-derived *E. ruminantium* include: (i) counting of organisms either by conventional phase contrast microscopy or by fluorescent microscopy, ii) measurement of *in vitro* infectivity using a plaque assay (TCLD50) (Totté et al., 1993), and iii) counting of infected cells in monolayer cultures after dye staining (Zweygarth et al., 2005). None of these methods are suitable for accurate quantitation of *E. ruminantium* in the blood of infected animals; they either lack sensitivity or are impractical for analysis of large numbers of samples.

PCR technology is widely used to aid in quantification of bacteria since the number of bacterial cells can be deduced accurately from the number of copies of target genes amplified by PCR. In quantitative PCR (QPCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule, ie. SYBR® Green, a double stranded DNA binding dye. The point at which the fluorescent signal is measured in order to calculate the initial template quantity can be either at the end of the reaction (endpoint QPCR) or while the amplification is still progressing (real-time PCR). Because the PCR reaction efficiency can decrease during later amplification cycles as reagents are consumed and inhibitors to the reaction accumulate, measurement of the template quantities based on the fluorescent signal during the exponential phase of amplification, as in real-time PCR, allows a more reproducible quantification than at the endpoint.

The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. The first cycle of amplification at which the generated fluorescence is above the background signal is called the threshold cycle (Ct). This Ct value can be directly correlated to the starting target concentration for the sample. When a standard curve dilution series is run alongside the unknown samples, the Ct values of the unknown samples are compared to those of the standard curve to determine the starting concentration of each unknown.

The PCR product can be verified by plotting fluorescence as a function of temperature to generate a melting curve graph of the amplicon. Because the melting temperature of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product and distinguish it from amplification artefacts that melt at lower temperatures.

This chapter describe the development of a quantitative real-time PCR suitable for quantification of *E. ruminantium* in the blood of infected sheep or infected cell cultures.

2.2 Materials and Methods

2.2.1 Growth and harvest of *E. ruminantium* in bovine endothelial cells.

Six *E. ruminantium* isolates were used: the Gardel isolate (both virulent and attenuated forms) from Guadeloupe in the Caribbean, the Welgevonden and Ball 3 isolates from South Africa, the Ghanaian Sankat 430 and Pokoase 417 isolates and the attenuated Senegal isolate from Senegal in West Africa (Table 2.1). Uninfected and *E. ruminantium*-infected bovine pulmonary artery (BPC) or bovine umbilical endothelial (BUE) cell cultures were maintained as described previously (Mutunga et al., 1998) and harvested for DNA extraction. Bacterial growth was monitored by microscopic examination of Giemsa-stained cytocentrifuge smears. Table 2.1 and 2.2 summarise the *E. ruminantium* isolates and cell lines used in this thesis.

2.2.2. Experimental infection of sheep.

Two stabilates each containing a different subpopulation of the Gardel isolate of *E. ruminantium* were used to infect sheep. The first stabilate consisted of a suspension of elementary bodies from endothelial cell cultures infected with *E. ruminantium* (CTVM Gardel stabilate at passage 15 *in vitro*, Bekker et al., 2005) in Glasgow minimal essential medium with 10% tryptose phosphate broth and 10% newborn calf serum (GMEM) cryopreserved with 10% added DMSO (STAB1, Bell-Sakyi et al., 2002). The second stabilate consisted of blood harvested from a sheep infected with *E. ruminantium* (Gardel, Uilenberg et al., 1985) cryopreserved with 10% DMSO (Utrecht University stabilate 366).

2.2.2.1. Experimental infection 1: Tick- and *E. ruminantium*-naïve cross-bred Suffolk sheep, aged between 6 and 9 months, were obtained from a commercial farm and held in isolation facilities at the Centre for Tropical Veterinary Medicine (CTVM), Edinburgh University, under Home Office regulations for animal experimentation. The sheep were fed on hay and concentrates and were treated with an anthelmintic (ivermectin) before the start of experimental procedures. Seven sheep (Annex 1) were inoculated intravenously with 1 ml of a 1:10 dilution of CTVM STAB1 in tissue culture medium; care was taken to administer the infected material within 13 minutes of thawing (Group 1). The sheep were monitored daily for rectal temperature and clinical signs (dullness, inappetance, rapid/deep breathing, nervous signs) and whole blood was collected in EDTA at 1-3 day intervals from day 0 (prior to inoculation) and throughout the infection period (Annex 1) and stored at -20ºC until required for DNA extraction. The sheep were euthanased on the third consecutive day of fever >40.8ºC or at the onset of severe clinical signs, whichever was sooner, and subjected to post-mortem examination for pathological changes characteristic of heartwater (hydrothorax, hydropericardium) and Giemsa-stained brain smears prepared for detection of *E. ruminantium.* The brain capillary cell infection rate was calculated by counting the number of *E. ruminantium* morulae per hundred brain capillary cell nuclei.

2.2.2.2. Experimental infection 2: Two 6-month-old female Texelaar sheep were used; #3154 was infected with 1 ml of a 1:10 dilution of CTVM STAB1 and #3464 with 2 ml of *E. ruminantium* blood stabilate CR366 respectively. Sheep were maintained in isolation under regulations of the Animal Ethics Committee of Utrecht

University, fed with hay and concentrates and treated with an anthelmintic (Ivomec) before starting the experiments. The sheep were monitored daily for rectal temperature and clinical signs and treated with tetracycline (Intramuscular Engemycine 10% at dose of 8 mg/kg body weight, max. 5 days) on the third consecutive day of fever. Whole blood and buffy coat (collected in EDTA) and serum samples were taken from these sheep before inoculation and daily for 15 days following infection and before starting antibiotic treatment (Annexes 9 and 12). Blood samples were stored at -20ºC until required for ELISA or DNA extraction.

2.2.3. DNA extraction. Total genomic DNA was extracted from *E. ruminantium*-infected endothelial cells using the QIAamp DNeasy tissue extraction kit (Westburg, Leusden, The Netherlands) while total DNA from whole blood (200 µl) or buffy coat (100 µl) was extracted using the QIAamp DNeasy blood extraction kit. Elution of DNA from the column was carried out using 100 µl of the elution buffer according to the manufacturer's instructions. DNA was stored at -20ºC until required.

2.2.4. PCR design. Five primers, designated F1, F2, F3, R1 and R2, were designed to amplify the *map1-1* gene of *E. ruminantium* (GenBank accession no. AY652746) using the DNA star program, and three different combinations of these primers were tested. Primer sequences were selected based on universal requirements for primer design and also on some specific criteria required for SYBR® Green real-time PCR assays: a target sequence that is unique in the genome and likely to be conserved among different *E. ruminantium* isolates; lack of features

predicted to result in dimer formation between primer pairs or self-annealing; selection of sites to produce small amplicons, ideally between 50 and 150 base pairs. Genomic DNA was used as template in a PCR reaction containing 1X Taq PCR buffer (Promega), 3 mM $MgCl_2$, 1.25 U of Taq polymerase (Promega), 400 µM of each deoxynucleoside triphosphate, 10 pmol of each primer in a 25 µl reaction. Reactions were carried out in an Engine Opticon thermacycler (MJ Research) or in a iCycler (Biorad) using the following standard programme (unless stated otherwise): 5 min at 95°C followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C and a final elongation step of 7 min at 72°C. PCR products were visualized by running the samples on agarose gels and staining with ethidium bromide. Table 2.3 shows the sequences of the primers and the size of PCR products generated from the different primer combinations used in this thesis.

2.2.5. Estimation of PCR sensitivity and specificity. The sensitivity of the PCR using the F3/R1 primer combination was estimated by amplifying dilutions of a purified plasmid pBAD/Myc-His (Invitrogen), containing the *map1-1* gene, in milliQ water. This construct will be hereafter referred as MAP1-1B, and was kindly provided by Omar Taoufik of Utrecht University. The number of plasmid copies (Y) was calculated using the following formula, in which X is the concentration of the plasmid DNA determined by spectrophotometry:

(Xg/µl DNA/ [plasmid length in basepairs x 660]) x $6.022x10^{23}$ = Y molecules/µl (http://www1.qiagen.com/literature/handbooks/PDF/PCRAndReverseTranscription)

Then a ten-fold dilution series was prepared (in duplicate) from the stock in milliQ water. One microliter of each dilution was submitted to PCR amplification in a 25 µl reaction mixture as described above except that the annealing temperature was decreased to 50°C.

To verify the specificity and ability of F3/R1 primers to amplify different isolates, gDNA extracted from uninfected bovine endothelial (BPC) cultures and non-infected sheep blood, along with gDNA from the *E. ruminantium* (Gardel virulent and attenuated, Welgevonden, Ball 3, Sankat 430, Pokoase 417 and attenuated Senegal isolates) derived from endothelial cell cultures, was submitted to PCR under standard conditions. PCR products were visualized by running the samples on agarose gels and staining with ethidium bromide.

2.2.6. Real-time PCR.

2.2.6.1. Experiment 1. A standard curve (1) was generated with data from PCR performed on three independent series of 3-fold dilutions in host DNA from a stock of *E. ruminantium* (Gardel) gDNA containing approximately 100 organisms per microlitre. The number of organisms present in 1 µl of culture supernatant was counted in Giemsa-stained preparations by light microscopy (Annex 13). Amplification of standards against a fixed concentration of DNA extracted from blood samples taken from non-infected sheep was done to test the influence of the host DNA on the recognition of the DNA from *E. ruminantium*.

2.2.6.2. Experiment 2. In an attempt to improve the accuracy of quantification, a second standard curve (2) was generated from data derived by performing PCR on three independent series of 10-fold serial dilutions of the MAP1-1B plasmid in milliQ water. The DNA concentration of the plasmid standard was determined by spectrophotometry and the approximate number of plasmid copies was calculated using the formula previously mentioned (section 2.2.5).

One or five microlitre samples from each standard dilution series (1 and 2) were submitted to PCR amplification in a 25 µl reaction mixture as follows: 12.5 µl of SYBR® Green supermix (Qiagen), 1 µl of F3 as forward primer, 1 µl of R1 as reverse primer (0.4 µmol/L final concentration), 1-5 µl of template, in a 25 µl PCR reaction. The cycling programme consisted of 95°C for 15 minutes followed by cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, repeated 40 times. The threshold cycle (Ct) values were determined and melting graphs analysed accordingly using the corresponding software.

Table 2.1

ISOLATE	GEOGRAPHIC AREA	REFERENCE
Gardel	Guadeloupe (Caribbean)	Uilenberg et al., 1985
Gardel attenuated	Guadeloupe (Caribbean)	Martinez, 1997
Gardel CTVM	Guadeloupe (Caribbean	Bekker et al., 2005
Senegal	Senegal (West Africa)	Jongejan et al., 1998
Senegal attenuated	Senegal (West Africa)	Jongejan, 1991
Welgevonden	South Africa	Du Plessis, 1985
Ball 3	South Africa	Haig., 1952
Pokoase 417	Ghana (West Africa)	Bell-Sakyi et al., 1997
Sankat 430	Ghana (West Africa)	Bell-Sakyi et al., 1997

Table 2.2

Cell line	ORIGIN	Reference
BPC	Bovine pulmonary artery endothelial cells	Mutunga et al., 1998
BUE	Bovine umbilical cord endothelial cells	Jongejan, 1991
AVL/CTVM13	*A. variegatum* larvae	Bell-Sakyi et al., 2000b
RAN/CTVM3	*R. appendiculatus* nymphs	Bekker et al., 2002
IDE8	*I. scapularis* eggs	Munderloh et al., 1994

Table 2.3 Primers used in the thesis

Gene	Name	Sequence 5' to 3'	Fragment size (bp)	Reference
16SrRNA	AnEhF1	GGTTTTGTCAAACTTGAGAG	507	This study
	AnEhR1	GTATTACCGCCGCTGCT		
16SrRNA	AnEhF2	AGAGTTTGATCCTGGCTCAG	470	This study
	AnEhR2	CGAGTTTGCCGGGACTT		
Tick Cyt C	Tick F	TAGAACTAGGCCAACCAGGAACAT	550	This study
	Tick R	AAAACCGGAAGAGAAAGTAAAAGT		
Bovine β-actin	Bov F	CCAGACAGCACTGTGTTGGC	270	T. Coffey, personal communication
	Bov R	GAGAAGCTGTGCTACGTCGC		
Map1-1	F3	TACGGTAAAGACTCTCCAACTAAA	182	This study
	R1	TATGCAGCTTCAATCTCTACTC		
Map1-1	F1	AAAAAGGAGGGTTCTAT	143	This study
	R1	TATGCAGCTTCAATCTCTACTC		
Map1-1	F2	ATACAACCCAAGCATACCACACT	237	This study
	R2	TATGCAGCTTCAATCTCTACTCTT		
Map1+1	F	TAATCCCCACCAATACCAGC	431	VanHerdeen et al., 2004
	R	GCTTCAGAAACAATCCCTGG		
Map1	F	CATTAGCGCAAAATACATGC	694	"
	R	GT(A/G)TTGCTGATGCAAAACCTGG		
Map1-1	F	CATTAGCGCAAAATACATGC	564	"
	R	AAAACCTGGATTGGCTACAG		
Map1-2	F	AATAAACTCATTGCAACAGGTATA	223	"
	R	ACCGATGTGCATCGTGTAGT		
Map1-3	F	GAAATCCAAATCCTGGACCT	599	"
	R	TGGTGCATTGTGTAAATTGG		

Gene	Name	Sequence	Fragment size	Reference
Map1-4	F	CCTCAGCATTTTACAACACCA	311	“
	R	CATCCGTTTAGGAGAACAGACA		
Map1-5	F	AATAGCGTCAACTTGCCTGTC	511	“
	R	TCACCAAAATGTGTTGTGGC		
Map1-6	F	TTTCTTGGAAGAACAGTGCGT	416	“
	R	TTTGATGCAGAAATCCCTGA		
Map1-7	F	TGTAGGTAGACGTGGCTGGC	302	“
	R	AGCTCCACAGGTTGAAGTACG		
Map1-8	F	CGCTAAAGAAAGCAACCTTC	327	“
	R	AAATCGTCTAACGCGAAATC		
Map1-9	F	CGGTTTTAGCGGAGCACTTGG	336	“
	R	GAAATCTCCGCCAATTCCTA		
Map1-10	F	TTACCAGCCAACTTAAGCCT	373	“
	R	GGGACTGCTGATGAATTACC		
Map1-11	F	TTTGCCTTTTCAACATTTCA	552	“
	R	GTCTCCACCAATACCGAAAC		
Map1-12	F	TACAAGCCAAGCATTTCGTA	656	“
	R	TTTGCTGATGATGAATCTGG		
Map1-13	F	CATTTCTGGTGCTTTAGGGT	422	“
	R	ACCCGTGGTAGTAACCTTCA		
Map1-14	F	TTCATCACCACTTCCTGTTG	771	“
	R	TCTTTTTCAAGCTCATGCTG		
Unknown	F	ATTAGCAGCACTCCCAATCC	234	“
	R	TGGAAAACAACACTTTTTGTGG		

Gene	Name	Sequence	Fragment size	Reference
16SrRNA	F	AGTGGGGAATATTGGACAA	603	This study
	R	GCGGTTGCATCGAATTAA		
23SrRNA	F	CGTGGTAGGCTGGCATAAG	408	This study
	R	TCACCTTTCCCTCACGGTA		
g-for	F	TAAT(A/G)TCATTA(A/G)TGT CATTTTTACC	815	Bekker et al., 2002
g-rev	R	A(A/T)(A/C/G/T)(C/T)AAA(C/T) CTT(A/C)(C/T)TCCAA(G/T)TTC		

2.3 Results

2.3.1. Experimental infection of sheep.

2.3.1.1. Experiment 1: The seven sheep developed patent heartwater in 11-14 days and were euthanased on the third day of fever. The *E. ruminantium* infection levels in their brain capillary endothelial cells ranged from <0.1% to 8%, increasing broadly relative to the length of prepatent period (Table 2.4).

2.3.1.2. Experiment 2: Sheep #3154 developed fever 11 days after inoculation and recovered rapidly after treatment on day 14. Sheep #3464 developed fever 13 days after inoculation. After the second treatment dose the fever had decreased considerably but the sheep died suddenly on day 16 post inoculation.

Table 2.4 Detection and quantification of *E. ruminantium* DNA by real-time PCR in whole blood from naïve sheep infected with mammalian cell-derived *E. ruminantium*[a]

Sheep No.	No. of *E. ruminantium* detected in blood on each day (D) [b]									1st day of fever	Brain cell infection rate [c]
	D0	D1	D4	D7	D10	D13	D14	D15	D16		
241	-	-	-	-	0.03	0.5				Day 11	<0.1%
489	-	-	-	-	-	0.13				Day 11	<1%
238	-	-	-	-	-	0.1	0.2			Day 12	<0.1%
147	-	0.02	-	-	-	0.7	Nd	95		Day 11	8%
546	-	-	-	-	-	-	Nd	0.8		Day 11	5%
391	-	-	-	-	-	-	Nd	Nd	38	Day 12	4%
651	-	-	-	-	-	0.1	Nd	Nd	3.5	Day 12	5%

[a] Blood samples were collected at 3-day intervals and just prior to euthanasia.

[b] The number of *E. ruminantium* determined by real-time PCR is expressed as the number of organisms per microlitre of blood and calculated with reference to a standard curve consisting of dilutions of organisms, obtained from *in vitro* endothelial cell cultures, counted by microscopy in Giemsa-stained EB preparations (section 2.2.6.1).

[c] Percentage of brain capillary endothelial cells infected with *E. ruminantium* in Giemsa-stained brain crushed smears prepared post-euthanasia.

2.3.2. PCR optimisation. To assess the efficacy of the different primer pairs, in terms of amount of PCR product and the absence of non-specific products, and to determine the optimal annealing temperature, the F1/R1, F2/R2 and F3/R1 primer combinations were used in a PCR to amplify DNA extracted from a positive control (*E. ruminantium* Gardel-infected endothelial cell cultures) at different annealing temperatures. All combinations of primers gave readily detectable amounts of amplicons free of non-specific products at temperatures between 53 and 56ºC (Fig. 2.1). However, when the same protocol was used on DNA from *E. ruminantium*-infected sheep blood, the F1/R1 and F2/R2 primer combinations yielded non-specific products and no signal was detected at temperatures above 53.4ºC (Fig. 2.2A). In contrast, the F3/R1 primer combination amplified specific products from both 3 and 5 microlitre aliquots of different DNA samples from *E. ruminantium*-infected sheep blood and yielded no non-specific products even at the lowest temperature tested, 52ºC (Fig. 2.2B). Therefore, the primer combination F3/R1 was selected for use in real-time PCR assays at an optimal temperature range of between 52º and 55ºC.

2.3.3. Estimation of PCR sensitivity and specificity. One microlitre from each standard dilution of plasmid DNA in milliQ water was submitted to amplification by PCR using the F3/R1 primers. A band of the expected size (182 bp) was observed at dilutions down to 10 plasmid copies per microlitre, in at least one of the duplicates (Fig. 2.3). Primers F3/R1 did not recognise DNA extracted from uninfected BPC or from uninfected sheep blood but did amplify DNA from five West and South African isolates (Senegal, Sankat 430, Pokoase 417, Ball 3 and Welgevonden) in addition to *E. ruminantium* (Gardel) (Fig. 2.4).

Fig. 2.1

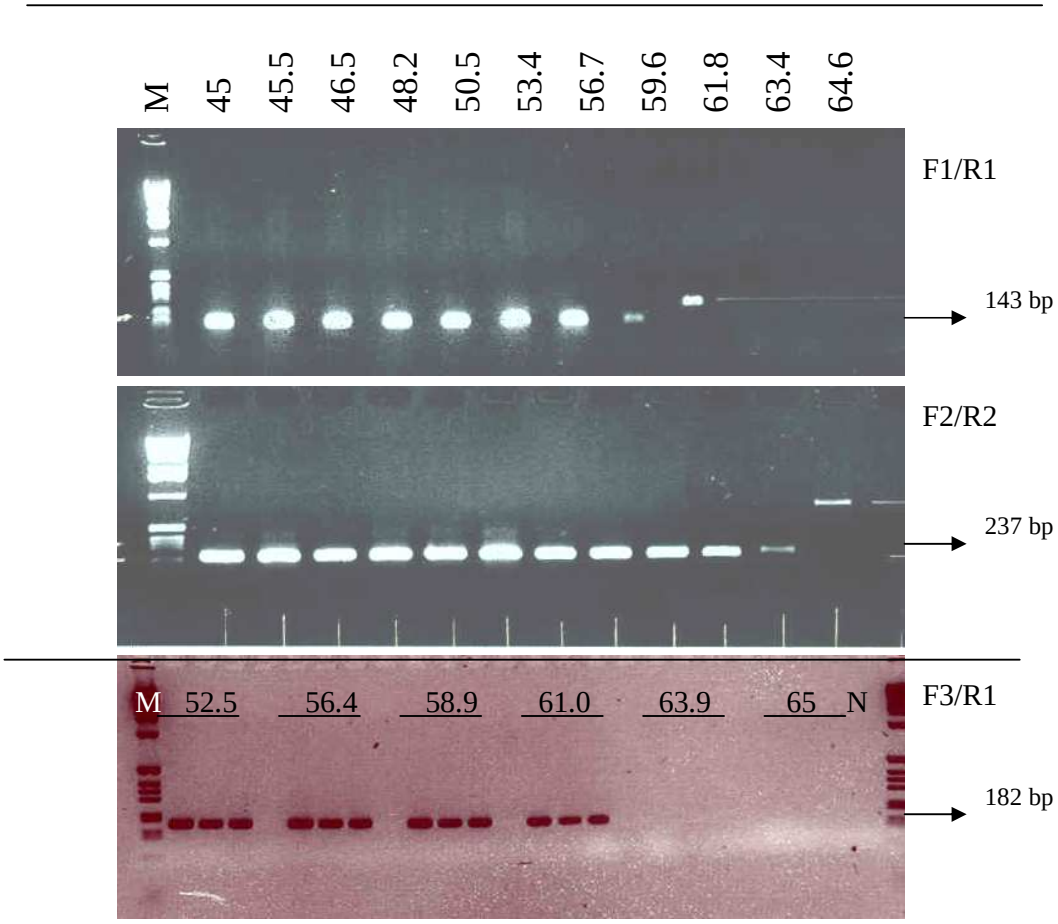


Fig. 2.1 Optimisation of annealing temperature for different combinations of primers using DNA extracted from *E. ruminantium*-derived endothelial cells as a positive control. Temperatures were controlled by a gradient temperature programme of the Engine Opticon thermocycler and are expressed in degrees centigrade. M: molecular marker. N: Negative control. 1.5 % agarose gel stained with ethidium bromide.

Fig. 2.2

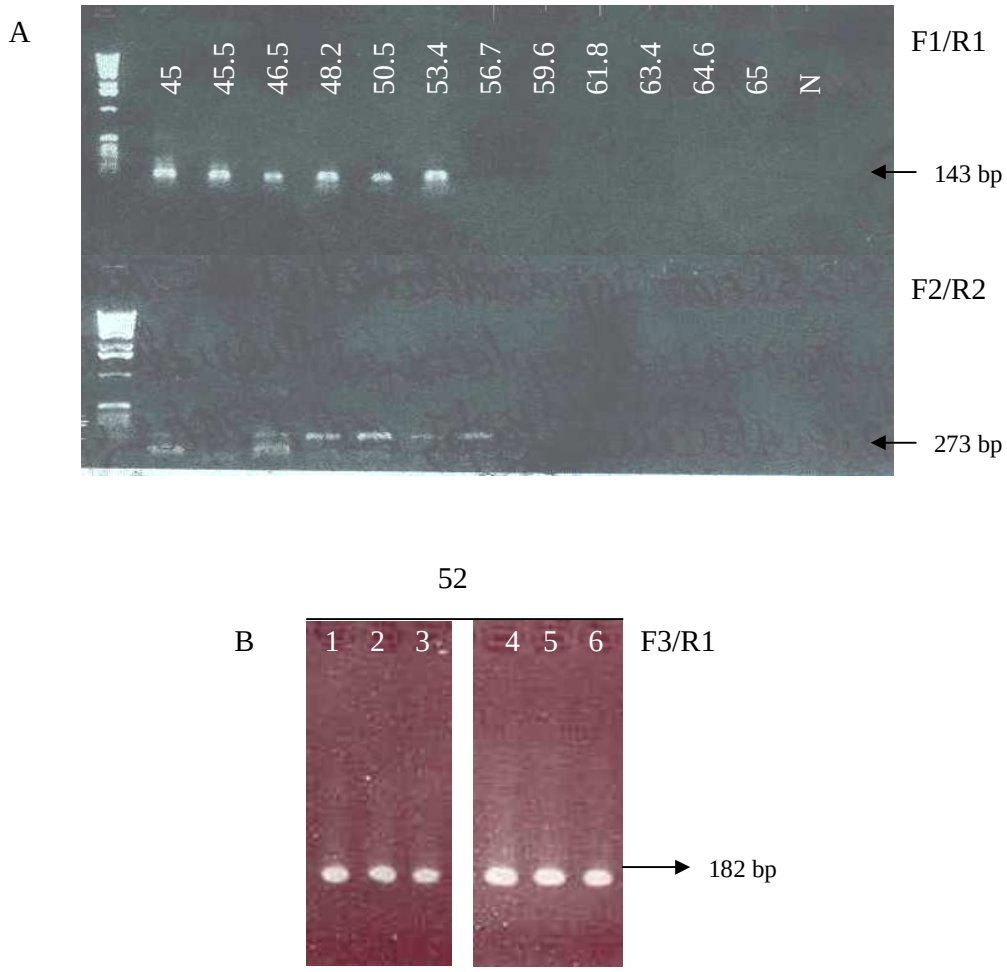


Fig. 2.2 Optimisation of annealing temperature for different combinations of primers using DNA extracted from *E. ruminantium* infected sheep blood. One (A, B: lanes 1, 2, 3) or five (B: lanes 4, 5, 6) microlitres of DNA were used. Temperatures were controlled by a gradient temperature programme of the Engine Opticon thermocycler and are expressed in degrees centigrade. M: molecular marker. N: Negative control. 1.5 % agarose gel stained with ethidium bromide.

Fig. 2.3

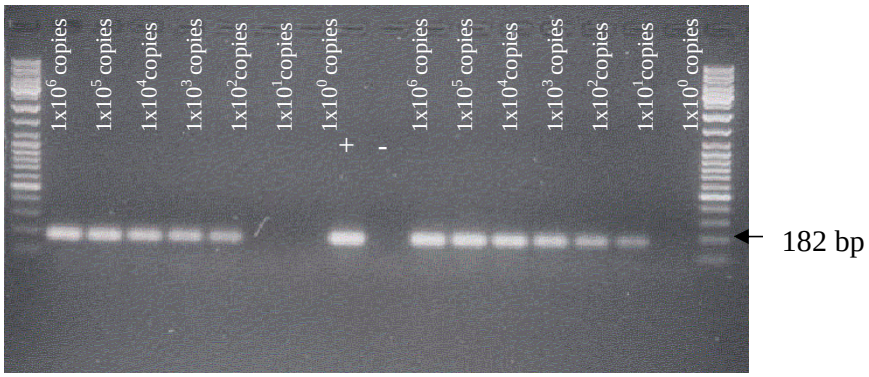


Fig. 2.3: Amplification by PCR of 10 fold-dilution samples (in duplicate) ranging from 10^0 to 10^6 copies of plasmid MAP1-1B using F3/R1 primers. +: gDNA positive control. –: negative template sample.

Fig. 2.4

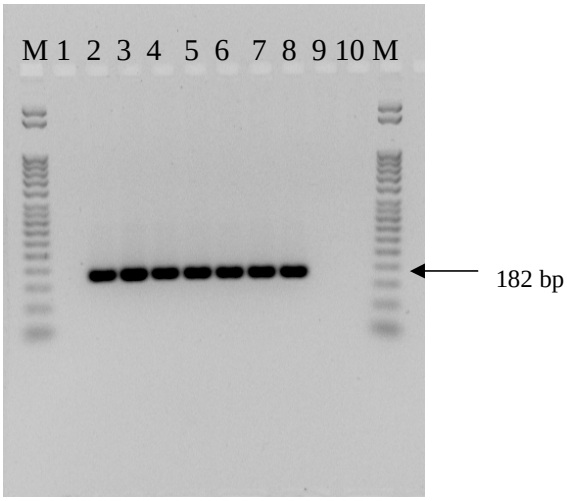


Fig. 2.4: PCR amplification of DNA from uninfected BPC (lane 1); uninfected sheep blood (9); *E. ruminantium* CTVM Gardel (2), attenuated Gardel (3), Ball 3 (4), Welgevonden (5), Sankat 430 (6), Pokoase 417 (7), attenuated Senegal (8) using F3/R1 primers. No-template control (10). M: 50-bp marker.

2.3.4. Estimation of the effect of background DNA. To test effects of host DNA on the efficiency of the PCR, a positive control sample containing 1 x 10^7 copies of pBAD plasmid containing the *map1-1* gene was used to prepare a ten-fold dilution series ranging from 10^6 to 1 copy/µl in deionised water, or in a fixed concentration of gDNA extracted from uninfected sheep blood or uninfected ticks. Then, 1 µl of each dilution sample was amplified by PCR using F3/R1 primers. A positive reaction was detected down to one copy/µl from DNA plasmid resuspended in water (Fig. 2.5A) or diluted in gDNA from uninfected sheep blood (Fig. 2.5B) or uninfected ticks (Fig. 2.5C), indicating that the sensitivity of the assay was not affected by host DNA.

2.3.5. Evaluation of DNA lost after extraction from blood samples. The proportion of specific DNA lost after extraction from sheep blood using DNA extraction kits (Qiagen) was evaluated by real-time PCR. A new series of 10-fold dilutions was prepared by diluting *E. ruminantium* (Gardel) gDNA in uninfected sheep blood; DNA was extracted from each of these samples yielding a final volume of 100 µl in elution buffer. Then 1 µl of the DNA suspension was used for PCR. Comparisons between the theoretical numbers of copies present in each sample versus the numbers of copies detected by real-time PCR diluted in buffer indicated a 10-fold reduction in sensitivity, indicating loss of the specific DNA during the extraction procedure from blood (Fig. 2.6 and Fig. 2.7).

Fig. 2.5

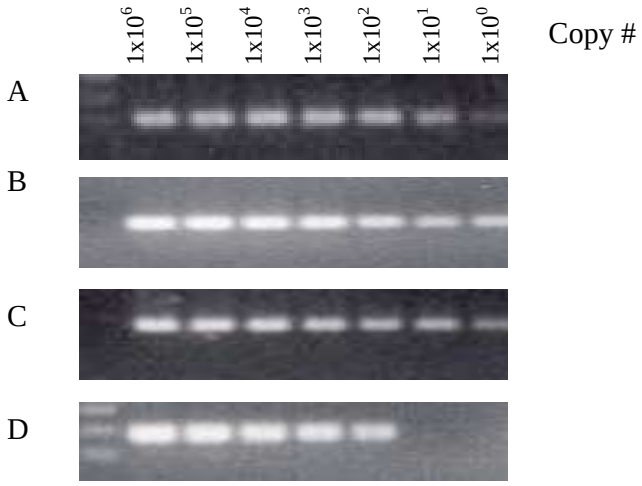
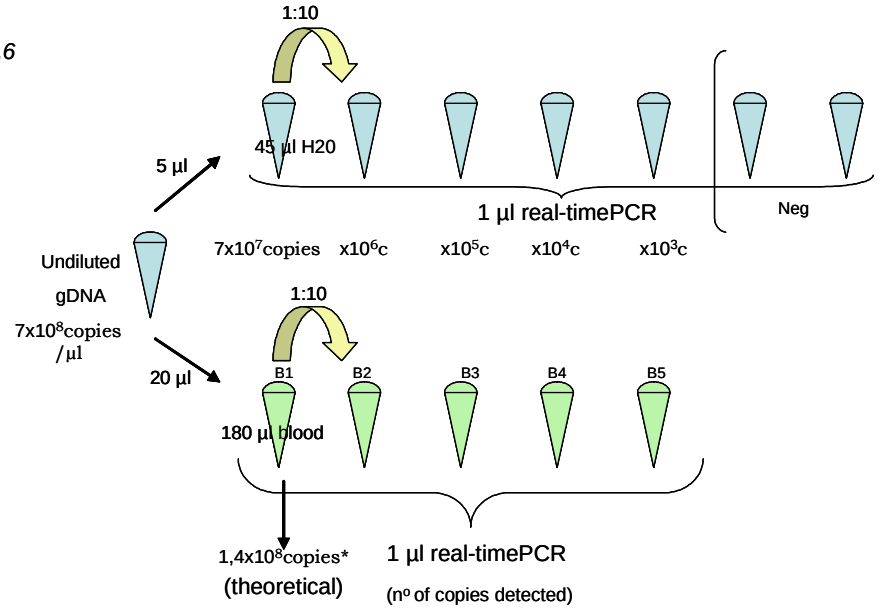


Fig. 2.5: Estimation of the effect of background DNA on amplification of MAP1-1B plasmid with primers F3/R1. Ten-fold dilution of plasmid copies in water (A), in DNA extracted from uninfected sheep blood (B), in DNA extracted from a pool of uninfected ticks (C) and in blood from an uninfected calf (D). Fragments are 182 bp.

Fig. 2.6



* $7x10^8$copies-----1 µl

X --------20 µl

$X=1,4x10^{10}$

$1,4x10^{10}$ -------200 µl=100 µl DNA elution

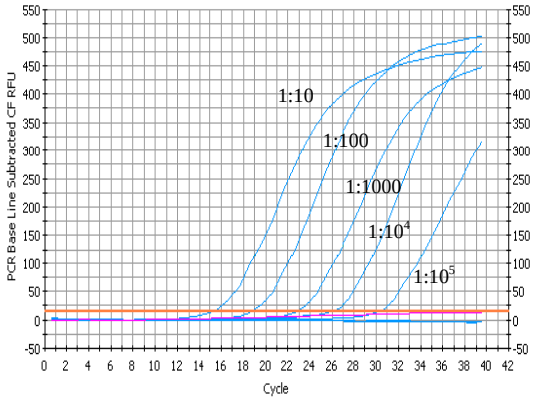
X----------1 µl DNA elution

$X=1,4x10^8$

Fig. 2.6 Diagrammatic representation of 10-fold dilutions of gDNA in water (A) and in uninfected sheep blood (B1-B5) subjected to DNA extraction and real-time PCR in order to estimate gDNA loss. The theoretical number of genome copies in the first blood dilution sample (B1) was calculated (*) and compared to the number of copies quantified by the real-time PCR (Fig. 2.7).

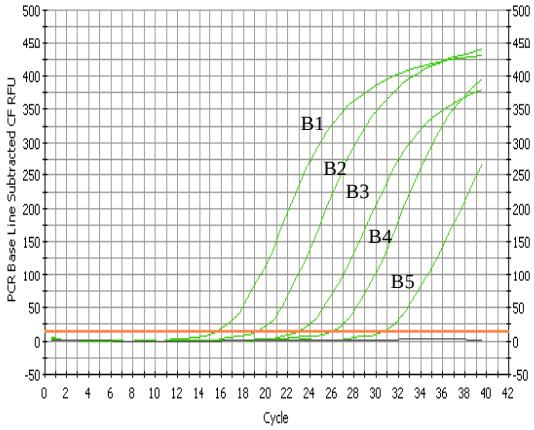
Fig. 2.7

A



gDNA dilutions	N° of copies detected by real-time PCR in gDNA/water dilutions
1:10	$7x10^7$
1:100	$4,8x10^6$
1:1000	$2,8x10^5$
$1:10^4$	$2,7x10^4$
$1:10^5$	$1,28x\ 10^3$
$1:10^6$	Neg
$1:10^7$	Neg

B



gDNA dilutions in uninfected blood	Theoretical N° of copies calculated in gDNA/blood dilutions if 100% recovery	No copies in gDNA/blood dilutions detected by real-time PCR
B1	$1,4x10^8$	$4,8x10^7$
B2	$1x10^7$	$3,7x10^6$
B3	$1x10^6$	$2,3x10^5$
B4	$1x10^5$	$2,7x10^4$
B5	$1x10^4$	$8,78x10^2$
B6	-	-
B7	-	-

Fig. 2.7: Effects of dilution of gDNA in water (A) and in uninfected sheep blood after DNA extraction (B) on the cycle threshold determined by real-time PCR. Quantities in corresponding tables express the number of copies of *E. ruminantium* in each dilution, calculated as in *Fig. 2.6* (theoretical) or detected by real-time PCR, per microlitre of DNA suspension.

2.3.6. Quantitation of *E. ruminantium* in positive controls.

To check if quantitation was reproducible between two different aliquots containing the same positive control preparation, two different aliquots of stabilate ER 06 (1 ml of supernatant from bovine endothelial cell cultures heavily infected with *E. ruminantium* Kerr Serigne, The Gambia; kindly provided by Bonto Faburay and cryopreserved with SPG in liquid nitrogen at Utrecht University) were submitted to DNA extraction, and from these DNA suspensions 1:10, 1:50 and 1:100 dilutions were prepared. Undiluted and diluted samples were submitted to real-time PCR. There was total agreement between the two aliquots in numbers of organisms present in both undiluted and diluted samples. Fig. 2.8 summarise the results.

2.3.7. Quantitation of *E. ruminantium* in sheep blood samples.

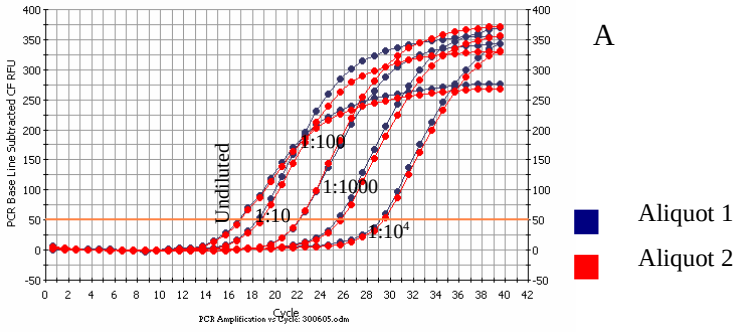
2.3.7.1. Experiment 1: DNA extracted from blood of the seven group 1 sheep was amplified by real-time PCR with F3/R1 primers. Amplification of the reference positive control DNA, based on counted organisms and subjected to 6 ten-fold dilutions showed linearity over the whole range (Fig 2.9B). Melting curve analysis was performed to check for specificity. Only one peak was identified in PCR products amplified from *E. ruminantium* DNA by the F3/R1 primers, indicating the presence of a single PCR product. There was no interference in the PCR reaction from non-specific products or primer dimers (Fig. 2.9C).

In the group 1 sheep, *E. ruminantium* DNA was detectable in all animals on the day of euthanasia and in some animals on previous days (Table 2.4). In blood from sheep 238, 147 and 651 *E. ruminantium* was detected on day 13 post inoculation,

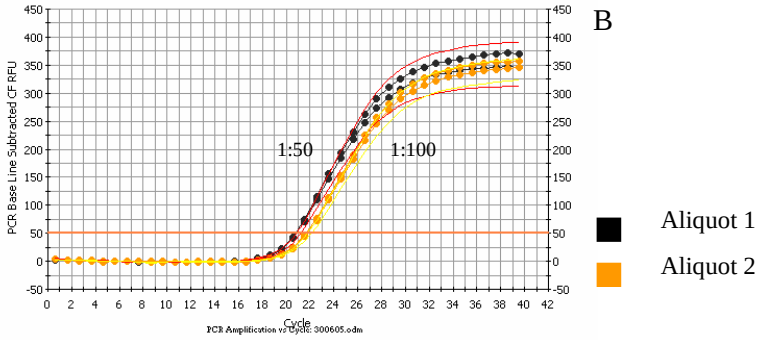
during the febrile reaction. However in sheep 241, a positive signal (estimated as 0.03 organisms) was detected on day 10 post inoculation, 1 day prior to fever in this animal. Positive results (estimated as 0.02 organisms) detected as early as day 1 post inoculation were considered to represent products of amplification of the inoculum itself.

Quantitation of the DNA extracted from the group 1 sheep indicated that levels of rickettsaemia were highest immediately before euthanasia, based on the standard curves (1) consisting of whole organisms quantified by microscopy and diluted in DNA host samples. Blood samples at this time were estimated to contain between 0.1 and 95 organisms/µl blood (Table 2.4). Negative controls did not give any fluorescence signal after 50 cycles of PCR.

Fig. 2.8



	Undiluted	1:10	1:100	1:1000	$1:10^4$
Aliquot 1	$1{,}2x10^8$	$4{,}1x10^7$	$4{,}1x10^6$	$5{,}3x10^5$	$4{,}7x10^4$
Aliquot 2	$1{,}1x10^8$	$3{,}4x10^7$	$4{,}3x10^6$	$4{,}26x10^5$	$3{,}9x10^4$



	1:50	1:50	1:100	1:100
Aliquot 1	$9{,}67x10^6$	$9{,}07x10^6$	$5{,}48x10^6$	$5{,}18x10^6$
Aliquot 2	$7{,}28x10^6$	$9{,}08x10^6$	$4{,}02x10^6$	$5{,}0x10^6$

Fig. 2.8: Effects of 10-fold (A) and 50- and 100-fold (B) dilutions of two different aliquots of gDNA in water on the cycle threshold determined by real-time PCR.

Fig. 2.9

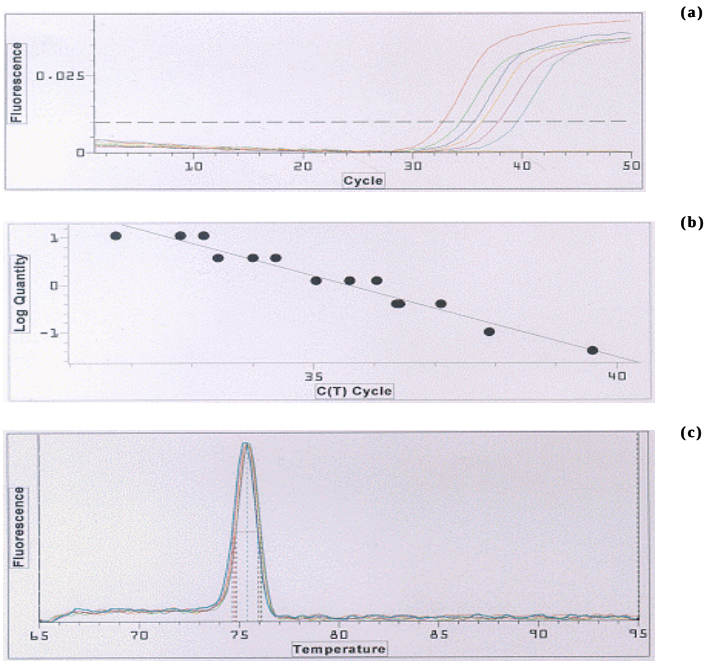


Fig. 2.9: (a) Quantitation graph showing the change in fluorescence of SYBR® Green dye plotted versus cycle number (one of each triplicate set shown). (b) Standard curve showing Ct values versus the log of the initial amount of gDNA (results of triplicate set). (c) Melting curve graph confirming amplification of the specific product. These analyses were carried out in an Opticon real-time thermocycler.

2.3.7.2. Experiment 2: In this experiment, DNA extracted from samples of both whole blood and buffy coat from the infected sheep were submitted to real-time PCR. A new set of samples was required since the samples used in the first experiment were no longer available. An alternative standard curve (2) was also generated in an attempt to obtain a more accurate quantification of *E. ruminantium* in blood. As with the standard curve (1) based on gDNA, the standard curve (2) generated with plasmid dilutions in water showed a linear correlation within the range analysed of 10^2-10^6 plasmid copies per PCR reaction. An identical melting temperature of 77.5ºC was obtained for the standard plasmids as well as samples containing *E. ruminantium* gDNA. A positive signal was only detected in those samples collected during the febrile response of the sheep. No major differences were observed between numbers of bacteria in whole blood and buffy coat samples during the 2 first days of fever. However on the third day of fever, between 3 and 6 times more organisms were detected in buffy coat samples in comparison with those of whole blood. Samples collected on days 6 and 9 post inoculation (prior to the onset of fever) and no-template negative controls did not yield any amplification after 40-45 cycles of PCR (Table 2.5). The corresponding amplification, standard curve, and DNA melting curve plots of this experiment are shown in Fig. 2.10.

Table 2.5 Detection and quantification of *E. ruminantium* DNA by real-time PCR in blood samples from naïve sheep infected with *E. ruminantium*[a]

		No. of *E. ruminantium* detected in blood on each day (D) [b]						
Sheep no.		D 6	D 9	D 11	D 12	D 13	D14	1st day of fever
3154	Blood	-	-	17.8	30.8	73.9	Nd	Day 11
	Buffy coat	-	-	86.4	37.8	646	Nd	
3464	Blood	-	-	Nd	104	87.4	393	Day 12
	Buffy coat	-	-	Nd	Nd	96.8	938	

[a] Blood samples were collected every day until the day of treatment from sheep infected by needle inoculation with STAB1 (3154) or stabilate CR366 (3464).

[b] The number of *E. ruminantium* determined by real-time PCR is expressed as the number of organisms per microlitre of blood and calculated with reference to a standard curve consisting of dilutions of MAP1-1B plasmids encoding the *map1-1* gene. (section 2.2.6.2).

Fig. 2.10

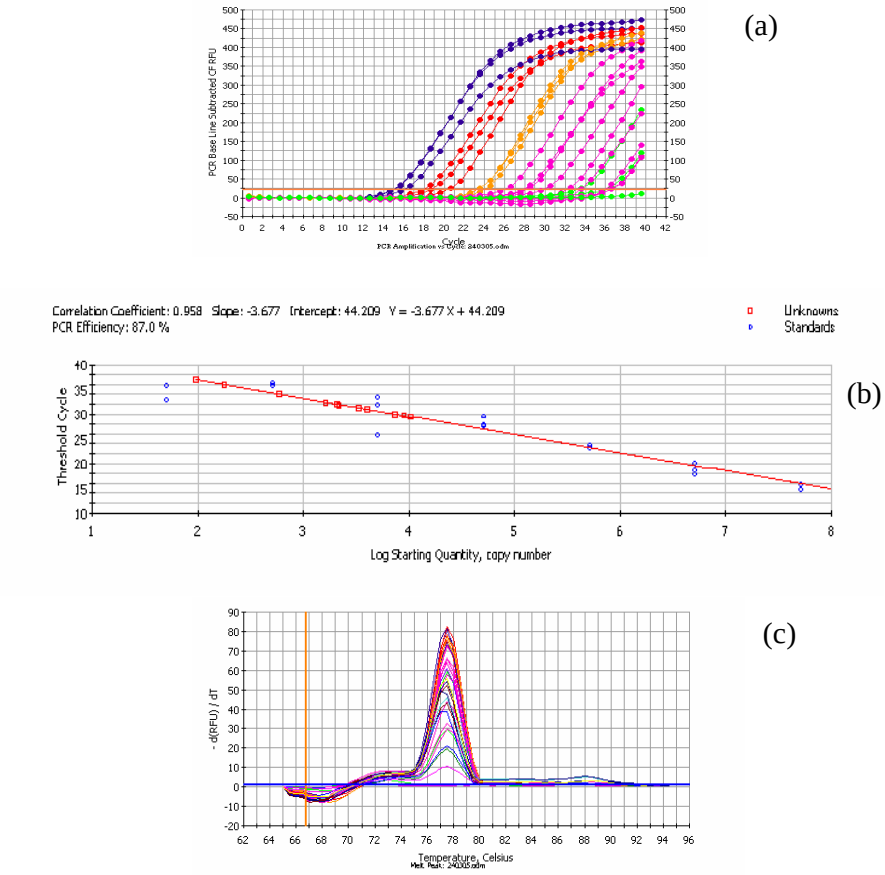


Fig. 2.10: (a) Quantitation graph showing the change in fluorescence of SYBR® Green dye plotted versus cycle number in triplicates (b) Standard curve showing Ct values versus the log of the initial amount of gDNA (results of triplicate set). (c) Melting curve graph confirming amplification of the specific product. These analyses were carried out in a Bio-Rad real-time thermocycler.

2.4 Discussion

This study aimed to evaluate the kinetics of *E. ruminantium* infection induced in naïve sheep after inoculation with virulent elementary bodies from endothelial cells or blood stabilates by attempting to quantify the numbers of bacteria in samples of peripheral blood.

Real-time PCR was chosen to quantify *E. ruminantium* in this study. As well as providing a more precise quantitive measure than conventional quantitative PCR, real-time PCR is regarded as more sensitive. A real-time PCR based on the use of SYBR® Green was employed. The advantage of using SYBR® Green in PCR is the increased sensitivity compared to ethidium bromide staining. The fluorescence intensity of SYBR® Green is enhanced over 100-fold on binding to DNA; in contrast, the fluorescence of ethidium bromide intensifies only 20-fold after binding to DNA (Seville, 2001; Garcia-Canas et al., 2002). The sensitivity of ethidium bromide has been estimated at about 5 ng of dsDNA by eye and about 600 pg using a CCD or Polaroid camera, while it is possible to detect less than 100 pg of SYBR® Green-stained DNA by eye and tens of picograms using a CCD system (Seville, 2001; Garcia-Canas et al., 2002). A disadvantage of any of these methods is that both specific and non-specific PCR products generate signals. Peixoto et al (2005) developed a SYBR® Green based real-time PCR protocol for quantitation of *E. ruminantium,* using primers that amplify the *map1* gene. However these primers also produced non-specific products from the bovine endothelial cell genomic DNA, although these were distinguishable from the *E. ruminantium* DNA target based on

different melting temperature. The authors rationalised that bulk *E. ruminantium* samples from *in vitro* cultures, such the ones they were handling, contained only trace amounts of endothelial cell DNA and therefore the non-specific products should not interfere. However, for quantitation of the small numbers of *E. ruminantium* that are expected in blood samples, even small amounts of nonspecific product could adversely affect the results. Care was, therefore, taken in selecting appropriate primers for use in the real-time PCR. The *E. ruminantium map1-1* gene was chosen as the target for PCR because it is the most conserved gene in the *map1* multigene family, thus allowing the detection of different *E. ruminantium* isolates. The combination of F3/R1 primers was selected, firstly because they generated no detectable non-specific products or primer dimers when used at the optimal annealing temperature and primer concentration, and secondly, because they were highly efficient, amplifying between 10 and 100 gene copies from dilutions of plasmid DNA. The F3/R1 primers amplified a band of the expected size when tested on DNA from 6 different isolates, but no bands were detected when DNA from uninfected sheep or uninfected endothelial cells was amplified. The presence of background DNA did not decrease the efficiency of the PCR assay as the same level of detection was achieved with DNA diluted in water and in sheep or tick gDNA. Analysis of DNA loss after extraction from a mixture of *E. ruminantium* gDNA and sheep blood indicated that there was an approximate 10-fold loss of the specific DNA in comparison with the calculated starting number of copies. Translating these findings to detection of organisms in infected blood, suggests that a minimum of 100 organisms would have to be present in 1 μl of peripheral blood in order to be detected with this assay: 100 bacteria/microlitre blood= 2×10^4 bacteria in the 200 μl

of blood used for DNA extraction= 2 x 10^3 in the 100 µl of DNA suspension after elution from the column considering the 10X DNA loss. As 1 µl was taken from this DNA suspension for PCR, then the number of organisms available for detection by PCR would be 20, just within the limit of detection. Therefore, it was considered that a potential way to increase sensitivity was to increase the amount of DNA in the PCR reaction. Five microlitre aliquots of DNA were therefore used in all the analyses in experiment 2.

A requirement for developing a real-time PCR is the generation of a standard curve that provides a realistic measure of the product in the samples to be analysed. In this study, the standard curve was produced by analysis of dilutions of DNA extracted from uninfected sheep blood to which DNA from a known number of *E. ruminantium* organisms was added. The addition of known numbers of organisms to blood prior to extraction would have been a more realistic measure, as it would have been possible to determine the efficiency of the releasing of DNA from whole organisms when they are diluted in blood. However, this was dependent on accurate quantitation of *E. ruminantium* and all studies described previously reported difficulties in defining the precise number of bacteria (Peixoto et al., 2005; Vachiery et al., 2006). In the present study, difficulties were encountered in quantifying elementary bodies from *E. ruminantium* cultures by light microscopic examination of Giemsa-stained preparations, including the small size of the bacteria (0.3-2.8 µm), the presence of nuclei from endothelial cells and aggregation of the bacteria. This led to an underestimate of bacterial numbers in the reference samples, giving unrealistically high levels of sensitivity; this was evident in some of the values cited from experiment 1, which estimated detection of 0.04 organisms using a standard

curve based on organism counts. Therefore a second approach was taken, in which dilutions of a purified plasmid, containing a copy of the *map1-1* gene (which is known to be a single copy gene, Collins et al., 2005), were used as external standards for DNA quantitation. This method was considered to be much more accurate and reproducible than quantitation by microscopy, as evident from results with the positive controls (section 2.3.6), although it might still over-estimate sensitivity in blood because of the potential loss of bacterial DNA during the lysis process.

E. ruminantium DNA was detected in all infected sheep on 1-3 days before the day of euthanasia/treatment, which correlated with the presence of clinical disease. Failure to detect *E. ruminantium* DNA in sheep blood during the prepatent period hindered the main aim of this chapter, namely to follow the development of infection during the pre-clinical phase. Nevertheless, relative quantitation of bacteria in blood during the clinical phase of infection was accomplished.

In previous studies, antigen of *E. ruminantium* was shown to be present in the bloodstream, as detected by ELISA, first in the plasma from day 3 to day 10 after experimental infection, then in the cellular fraction between days 5 and 21, at a higher concentration in a red blood cell fraction than in a white blood cell fraction (Neitz et al., 1986). The infectivity of the red blood cell fraction was found to be due to the presence of infected neutrophils trapped in the red cell fraction during centrifugation of the blood (Logan et al., 1987). In addition, Mahan et al. (1992), using DNA probe hybridisations, were able to detect *E. ruminantium* from sheep before the onset of fever in 5-10 ml of plasma. Enhanced signals were detected by increasing the amount of plasma used to prepare the target DNA. The present study, however, consistently failed to detect *E. ruminantium* DNA in 200 µl of whole blood

or 100 µl of buffy coat samples collected from infected, previously naïve, sheep prior to the onset of clinical disease. This may have been to either to very low levels of bacteria being present during the incubation period combined with the small sample tested in the PCR (ie. only 1% of the DNA extracted from the 200 µl of blood was used in the PCR assay) or failure to release bacterial DNA during the lysis of organisms in whole blood. Enrichment procedures that derive DNA samples from larger volumes of blood appear to be required for successful detection of the small numbers of *E. ruminantium* present in animals prior to the febrile reaction.

In conclusion, quantification by real-time PCR of *E. ruminantium* is possible in blood from clinically reacting sheep, and has advantages over traditional PCR methods, which only detect whether samples are positive or negative. However, the sensitivity of the whole system must be improved. One possibility is to incorporate a step for enrichment of *E. ruminantium* DNA before DNA extraction from larger volumes of blood (Sirigireddy and Ganta, 2005).

Chapter 3: Study of the *in vitro* transcriptional activity of the *map1* multigene family of *E. ruminantium*

3.1 Introduction

Successful *in vitro* cultivation of *E. ruminantium* using mammalian endothelial cells was described for the first time by Bezuidenhout et al (1985), while systems for the propagation of *E. ruminantium* in tick cell lines have become available only recently (Bell-Sakyi et al., 2000a; Bell-Sakyi, 2004). Studies using these *in vitro* systems have shown that there are clear differences in morphology between bacteria grown in endothelial cell and tick cell cultures (Bell-Sakyi et al., 2000b), and also in their immunogenicity and pathogenicity for sheep (Bell-Sakyi et al., 2002). Together these data suggest that *E. ruminantium* grown in endothelial and tick cell cultures are likely to differ in gene transcription and protein expression.

Recent studies of transcriptional activity of paralogs of the *E. ruminantium map1* multigene family have demonstrated that differences in the transcription of these genes exist between organisms grown in cultured host and vector cell environments. All 16 paralogs were transcriptionally active when *E. ruminantium* organisms were grown in endothelial cells (van Heerden et al., 2004) while in *E. ruminantium*-infected tick cell lines between 4 and 11 paralogs were found to be transcribed (Bekker et al., 2005). However, the triggers for this differential transcription have been little investigated.

Environmental stimuli such as temperature, pH, cell density, and host-specific factors appear to work in concert to affect the switch in expression of proteins in *B. burgdorferi* (Roberts et al., 2002). Amongst these, temperature has been demonstrated to be an important factor in controlling protein regulation (Schwan and Piesman, 2002). Outer surface protein (Osp) A is a major surface protein that is

abundant in *B. burgdorferi* spirochaetes grown *in vitro* and is also detectable in unfed infected ticks. A change in the *in vitro* growth temperature from 24ºC to 37ºC stimulated the spirochaetes to synthesise a different protein, OspC, whereas decreasing the temperature back to 24ºC caused OspC to be diminished to below detectable levels (Schwan, 1995). Zhi et al. (2002) also reported temperature as one of the factors that influence the expression of members of the *A. phagocytophilum p44* multigene family. Both mRNA and protein produced from the *p44-18* gene were detected in *A. phagocytophilum* cultivated in HL-60 cells at 37ºC, but their expression levels decreased in organisms cultivated in the same cells at 24ºC. Furthermore, the expression of the *p44-1* gene was inversely affected by temperature and *p44-1* transcripts were detected in cell cultures at 24ºC but not at 37ºC. Unver et al (2001) compared the transcription of *p30* paralogs in *E. canis* cultivated in the DH82 canine monocyte cell line at 37ºC and 25ºC to serve as a temperature model for dog and tick infections, respectively. Downregulation of the *p30* paralogs, except for *p30-10,* in ticks as well as in DH82 cells grown at 25ºC suggests that temperature may be one of the factors regulating the mRNA expression of *p30* paralogs.

Previous studies of *E. ruminantium,* have suggested that temperature may not play a role in the transcription of the *E. ruminantium map1* multigene family *in vitro,* as the same *map1* paralogs transcribed at 37ºC in AVL/CTVM13 cells were also detected at 31ºC in IDE8 cells (Bekker et al., 2005); moreover, no differences were seen in transcription patterns of *E. ruminantium* grown in different endothelial cell lines at 30ºC or 37ºC (van Heerden et al., 2004). However, no attempt has been made to investigate if there are differences in transcription between different developmental stages of the organism within a single host cell system. Based on electron

microscopy studies, three morphologically distinguishable developmental forms of *E. ruminantium* were identified in endothelial cell cultures (Jongejan et al, 1991c). The first and second forms, detected between days 2 and 5 p.i., were the reticulated and intermediate bodies, which the authors considered to be the vegetative, non-infectious forms of the bacteria. Electron-dense elementary bodies, considered to be the infective forms of the bacteria, were found between days 5 and 6 p.i. corresponding to the final stage of the infection. Recently, Marcelino et al (2005) reported on the *E. ruminantium* growth kinetics in an infection cycle of 6 days in BAE cells. In this study, when a bacterial suspension was collected before 108 hours post-infection (hpi) (4.5 days) and used to infect a fresh culture, very low levels of infection were produced resulting in low yields of *E. ruminantium*. The authors attributed this finding to the non-infectious character of intracellular reticulate bodies (RBs) at this stage of development. In contrast, at 120 hpi (5 days) the ability of *E. ruminantium* to infect new monolayers was markedly increased and this time point was defined as optimal for harvesting infective forms from *E. ruminantium* cultures. This finding reflected the presence of large numbers of infectious particles in cultures after 5 days of infection.

The aims of the work described in this chapter are twofold: firstly, to further investigate if temperature plays a role in the transcription of the *E. ruminantium map1* multigene family *in vitro*, and secondly, to investigate whether different developmental forms of *E. ruminantium* exhibit differential transcription of the *map1* cluster. Analysis of the transcriptional activity of *map1* paralogs of *E. ruminantium* grown in three different tick cell lines, each at 31ºC and 37ºC and *E. ruminantium* (Gardel isolate) cultured in bovine pulmonary artherial endothelial cells (BPC) also

at 31ºC and 37ºC was carried out. The transcriptional activities of *map1* paralogs in early or late stages and from different fractions of *E. ruminantium*-infected endothelial cell cultures maintained at 37°C were also compared.

3.2 Materials and Methods

3.2.1 Growth and harvest of *E. ruminantium* in bovine endothelial cells.

To test the effect of temperature on transcription, the CTVM Gardel isolate of *E. ruminantium* (Uilenberg et al., 1985, Bekker et al., 2005) was cultured in bovine pulmonary arterial endothelial cells (BPC) at 31ºC and 37ºC as described previously (Mutunga et al., 1998). Bacterial growth was monitored by microscopic observation of organisms in Giemsa-stained cytocentrifuge smears. When cytolysis of *E. ruminantium*-infected BPC cultures reached about 90% due to infection, cells were scraped from the bottom of the culture flasks and the whole cultures (scraped cells plus supernatant) were pelleted by centrifugation at 15,000 x g for 20 min at 4ºC. The pellets were immediately submitted to RNA extraction.

For the time-course study, the IBET Gardel isolate was grown in bovine umbilical endothelial (BUE) cells. In an initial experiment, 5ml of an infected culture (scraped cells plus supernatant) was passed 10 times through a 26G syringe needle to disrupt the cells and bacterial colonies. Then, aliquots of 1.25 ml were dispensed into 4 $25cm^2$ flasks containing uninfected BUE cells. After 5 hours of incubation, the cell monolayers were washed 3 times with medium and finally fresh medium was added to each flask. Whole cultures (cells plus supernatant) were harvested from one flask on each of days 1, 2, 3 and 4 p.i. and RNA was extracted.

For a second experiment, 1ml of supernatant medium from a culture of *E. ruminantium* Gardel/IBET in BUE cells exhibiting about 90% cytolysis was used to infect each of six $25cm^2$ flasks of uninfected BUE cells at 37ºC. After 24 hours of

incubation, the cultures were washed 3 times as above and fresh medium was added to each flask. After 3 days, the supernatant medium was discarded from 3 flasks, the monolayer was washed once with medium and the cells were scraped from the bottom of the flasks, spun at 200 x g for 5 min at 4ºC and stored in lysis buffer (RLT buffer) at –80ºC until RNA extraction. On day 6, when the cultures showed about 90% cytolysis, only supernatant medium was collected from the remaining three flasks and spun at 15,000 x g for 20 min at 4ºC, and the pellets were stored in RLT buffer at –80ºC until RNA extraction.

3.2.2 Growth and harvest of *E. ruminantium* in tick cells. The CTVM Gardel isolate of *E. ruminantium* was cultured *in vitro* at 31ºC in three tick cell lines, AVL/CTVM13, IDE8 and RAN/CTVM3 as described previously (Bell-Sakyi et al., 2000a,b; Bekker et al., 2002). The tick host species from which these cell lines were derived are described in Table 2.2 (All the tick cell cultures used in this study was kindly provided by Dr. Lesley Bell-Sakyi). Bacterial growth was monitored in Giemsa-stained cytocentrifuge smears. For this study, *E. ruminantium* was also cultured in AVL/CTVM13 at 37ºC. Attempts to adapt RAN/CTVM3 and IDE8 to grow continuously at 37°C were unsuccessful. Nevertheless, RAN/CTVM3 and IDE8-infected cultures at 31°C were transferred to 37°C after 4 and 5 days of infection respectively and maintained at this higher temperature for a week before harvesting. Cultures harvested by collection of adherent cells plus supernatant medium were spun at 200 x g for 5 min at 4°C and the pellets were immediately submitted to RNA extraction.

3.2.3 RNA and DNA isolation. Total RNA and DNA from *E. ruminantium*-infected endothelial and tick cell cultures were extracted using the tissue protocol provided with the QIAamp extraction kit (Westburg). Total RNA was treated with DNases (Promega) after elution from the column following the manufacturer's instructions.

3.2.4 cDNA synthesis and paralog specific PCR. First, DNase-treated RNA was checked for DNA contamination by synthesis of cDNA using forward and reverse PCR primers specific to the *E. ruminantium* 16S ribosomal gene under the same conditions described below. Control samples were prepared by omitting reverse transcriptase from the reaction. Sequences of 16S ribosomal primers are presented in Table 2.3.

Then, 10 µl of RNA samples, previously checked to be free from gDNA contamination, were used to prepare 40 µl of cDNA using 100 ng of random hexamer primers of the SuperScript^{TM} first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Two microliters of cDNA was subsequently used as template in a PCR reaction containing: 1x Taq PCR buffer (Promega), 3 mM $MgCl_2$, 1.25 U of Taq polymerase (Promega), 400 µM of each deoxynucleoside triphosphate, 10 pmol of each MAP1 family specific primer (Table 2.3) in a 25 µl reaction. Reactions were carried out on a iCycler (Biorad) using the following programme: 2 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C and a final elongation step of 7 min at 72°C. Positive controls included genomic DNA instead of cDNA. PCR products were visualized by running the samples on ethidium bromide-stained agarose gels.

3.3 Results

3.3.1 Transcription of the *map1* multigene family of *E. ruminantium* in endothelial cell cultures at different temperatures.

RT-PCR products obtained after amplification with 16S ribosomal primers gave a fragment of the expected size (approx. 500 bp), although less intensity was observed in the band corresponding to the culture maintained at 31ºC. Bands were observed in all samples except negative control samples (without reverse transcriptase). This result shows that *E. ruminantium* RNA was present in all the samples, and indicates that the RNA was free from gDNA contamination (Fig.3.1). To demonstrate the efficiency of the primers to amplify all paralogs, including *map1-2*, gDNA from IBET Gardel was amplified with all combinations of primers giving a fragment of the expected size with each pair. On some occasions, gDNA from CTVM Gardel was included in the analyses, which gave a band with all combinations except for the *map1-2* forward and reverse primers (Fig. 3.2A).

In *E. ruminantium* (CTVM Gardel)-infected BPC maintained at 37ºC, transcripts for 15 of the 16 *map1* paralogs were detected using paralog-specific PCR primers; *map1-2*, which is missing from the CTVM Gardel genome (Bekker et al, 2005), was not detected. The bands corresponding to the *map1* and *map1-8* genes seemed to be more intense than the ones obtained from gDNA and appeared to have more abundant transcripts when compared with the other genes. Also bands from paralogs *map1-5* and *1-14* showed slightly less intensity in comparison with the other paralogs. In the same cultures maintained at 31ºC, transcripts for 11 of the 16 *map1*

paralogs were detected. Among the genes transcribed an overall decrease in intensity of the RT-PCR products compared to those obtained from mammalian cells cultured at 37°C was observed. The band corresponding to the *map1-8* gene seemed to have more abundant transcripts whereas *map1-7, 1-9* and *1-10* seemed to have less, as judged by the intensity of the bands. No transcripts were detected for *map1-3, 1-5, 1-12, UN* and *1-14* genes (Fig. 3.2). Results of the analysis of transcription in endothelial cells at both temperatures are summarised in Table 3.1, and the variation in the amount of RT-PCR products indicated for individual paralogs.

One important observation in relation to interpretation of the PCR results, is that the amount of *E. ruminantium* in cultures at 31°C appeared to be much lower than in the cultures maintained at 37°C as confirmed by examination of Giemsa-stained cytocentrifuge smears.

Fig. 3.1

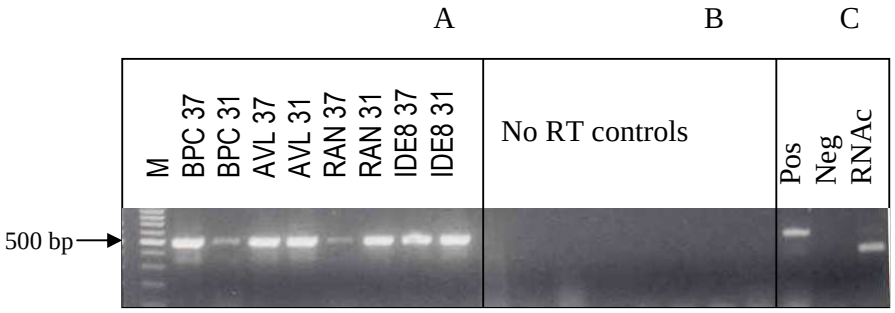


Fig. 3.1: Detection of 16S ribosomal RNA transcripts from *E. ruminantium* (CTVM Gardel)-infected BPC, AVL/CTVM13 (AVL), RAN/CTVM3 (RAN) and IDE8 cultures incubated at 31°C and 37°C (A). No reverse transcriptase negative controls from same samples (B). Pos: *E. ruminantium* DNA positive control; Neg: no template negative control; RNAc: Commercial RNA as positive control for RT-PCR (C). M: Molecular marker. PCR products were run on 1.5 % agarose gel stained with ethidium bromide.

Fig. 3.2

map1 genes

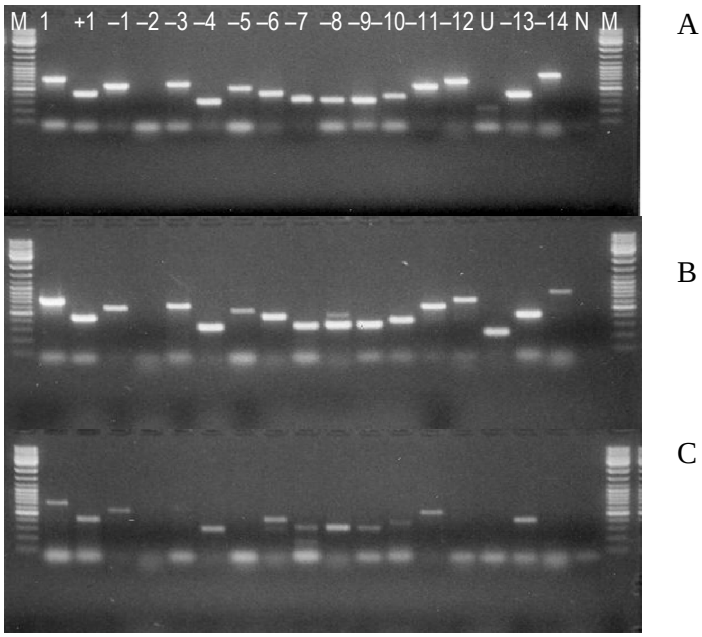


Fig. 3.2: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in BPC. Lanes 1 to -14 contain PCR products, all amplified with the respective *map1* cluster specific primer pairs shown above, from (A) gDNA (positive control); (B) cDNA from *E. ruminantium* cultured at 37˚C and (C) cDNA from *E. ruminantium* at 31˚C. N: no template negative control. M: molecular marker.

3.3.2 Transcriptional analysis of the *map1* gene cluster in tick cell cultures at different temperatures.

Figure 3.3 shows the results obtained from the vector cell line AVL/CTVM13 infected with *E. ruminantium* (CTVM Gardel) and incubated at 31ºC or 37ºC. At 31ºC, transcripts were detected for all paralogs except *1-3, 1-5, 1-6 and 1-12*. However, in cultures maintained at 37ºC fewer paralogs were detected; thus in addition to *1-3, 1-5, 1-6 and 1-12*, *map1-4, 1-9, 1-10 and 1-11* were also not detected. The overall intensity of RT-PCR products was reduced in cultures at 37ºC with the exception of paralogs *map1*, *map1+1* and *map1-1* for which the RT-PCR products showed the same intensity in cultures at both temperatures. In infected cultures of the non-vector line IDE8 maintained at 31ºC, the pattern of transcription was very similar to that observed in AVL/CTVM13 at 31ºC, in which paralogs *1-3, 1-5, 1-6, 1-12* were not detected; however, *map1-10* was also not detected (Fig. 3.4). In infected RAN/CTVM3 at 31ºC, only 3 bands of the expected size were detected for paralogs *map1+1, map1* and *map1-1* (Fig. 3.5). In both the non-vector lines RAN/CTVM3 and IDE8, grown at 37ºC for 7 days, there was virtually no transcriptional activity for the *map1* cluster; in both cases only a faint signal for paralog *map1-1* was observed (Figs. 3.4 and 3.5). In general, for all tick cell lines tested at 31ºC and for AVL/CTVM13 at 37ºC, the transcript for *map1-1* was present and was the predominant transcript. Transcripts for *map1-3*, *map1-5*, *map1-6* and *map1-12* were never observed in any of the infected tick cell lines. Results of the transcription are summarised in Table 3.2 in which the variation in the intensity of the PCR product obtained for the individual paralogs is indicated.

As confirmed by examination of Giemsa-stained cytocentrifuge smears, all tick cell cultures maintained at 37°C showed apparent lower levels of bacteria compared with cultures grown at 31°C. Thus, the proportion of *E. ruminantium* message in the RNA preparations might be expected to be lower than in the 31°C cultures.

Fig. 3.3

map1 genes

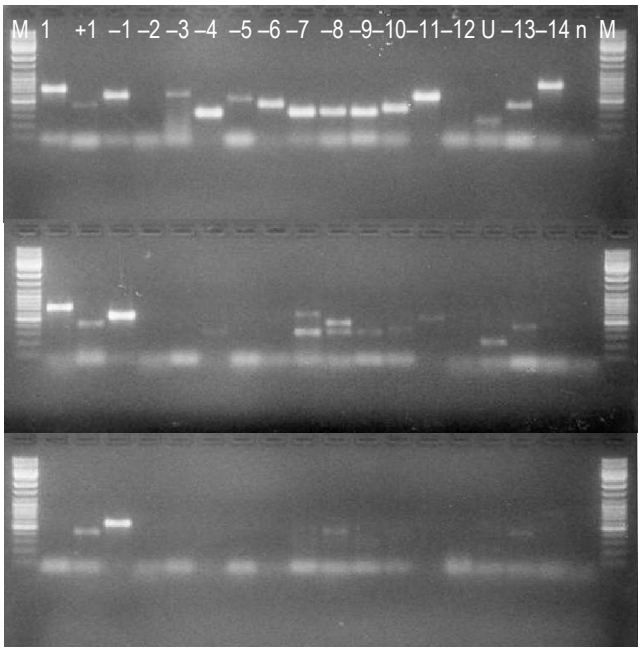


Fig. 3.3: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in AVL/CTVM13. Lanes 1 to -14 contain PCR products, all amplified with the respective *map1* cluster specific primer pairs shown above, from (A) gDNA (positive control); (B) cDNA from *E. ruminantium* cultured at 31˚C and (C) cDNA from *E. ruminantium* at 37˚C. N: no template negative control. M: molecular marker.

Fig. 3.4

map1 genes

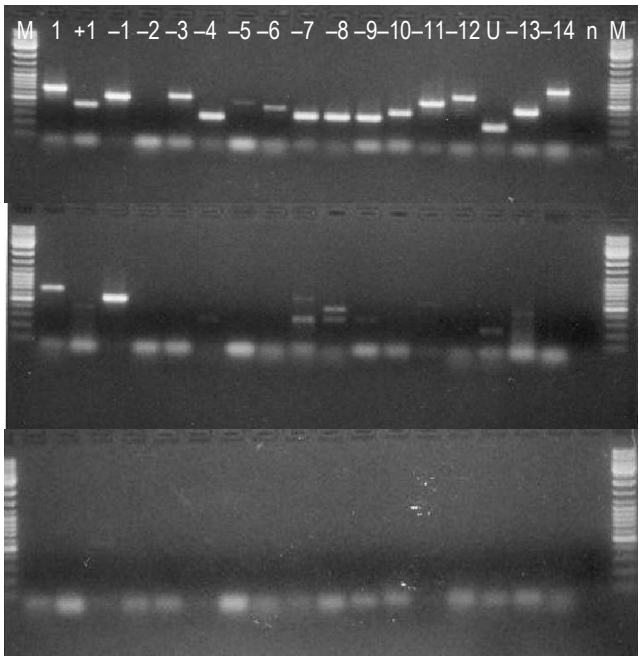


Fig. 3.4: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in IDE8. Lanes 1 to -14 contain PCR products, all amplified with the respective *map1* cluster specific primer pairs shown above, from (A) gDNA (positive control); (B) cDNA from *E. ruminantium* cultured at 31˚C and (C) cDNA from *E. ruminantium* at 37˚C. N: no template negative control. M: molecular marker.

Fig. 3.5

map1 genes

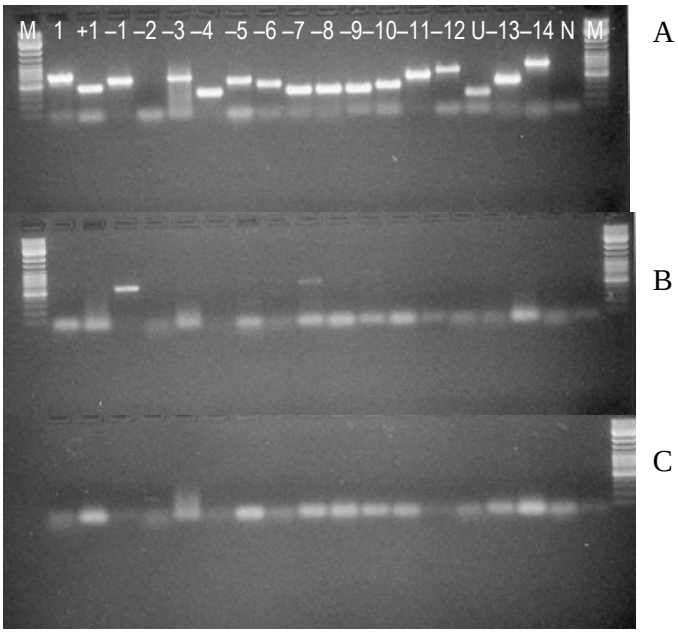


Fig. 3.5: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in RAN/CTVM3. Lanes 1 to -14 contain PCR products, all amplified with the respective *map1* cluster specific primer pairs shown above, from (A) gDNA (positive control), (B) cDNA from *E. ruminantium* grown at 31˚C and (C) cDNA from *E. ruminantium* cultured at 37˚C. N: no template negative control. M: molecular marker.

3.3.3 Transcription of the *map1* cluster of *E. ruminantium* in endothelial cells on different days after infection.

In the first experiment of the time-course study all *map1* paralogs were found to be transcribed in samples from days 1 and 2 after infection. However, paralogs *1-3, 1-5 and 1-6* were not detected on d3 p.i, and paralog *1-14* was not detected in the d4 p.i. sample (Fig. 3.6), when the culture exhibited approximately 90% of cytolysis. As this experiment was carried out with samples comprising whole cultures (cells plus supernatant) and therefore more likely to contain a mixture of developmental forms, the second experiment was designed in order to favour the detection of transcripts from stages within intact cells or free organisms in supernatant only. RT-PCR products from triplicate samples collected on days 3 and 6 p.i. were compared (Fig. 3.7 and 3.8). Detection of message for a paralog in at least one of the triplicate samples examined was considered to be a positive result, as all *map1* primers had been shown to be efficient at generating specific fragments when gDNA was used as a template. Transcripts from all paralogs were detected in samples collected on d3 p.i., while on d6 p.i., when Giemsa-stained cytocentrifuge smears showed that the samples contained high levels of EBs, all paralogs except *1-5, 1-12 and 1-14* were detected.

Fig. 3.6

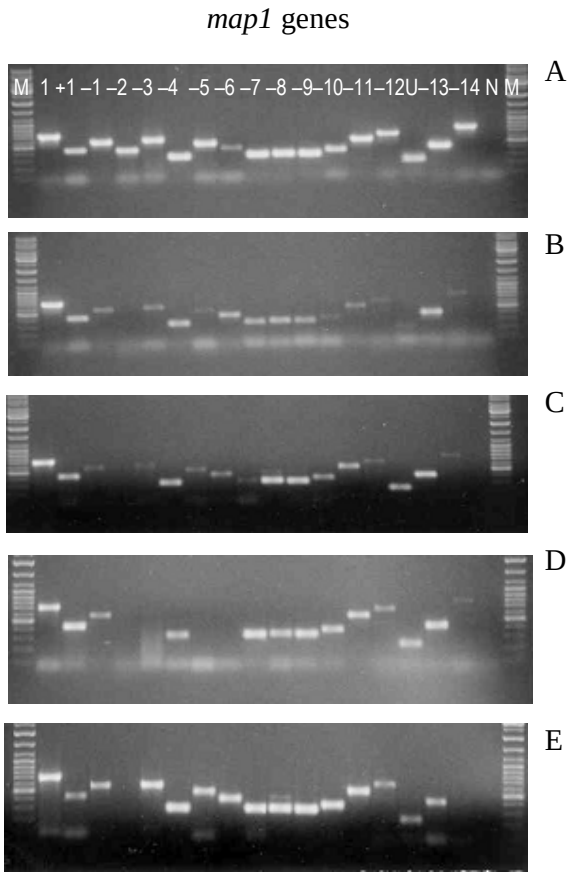


Fig. 3.6: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in endothelial cells (BUE) at different days post infection (dpi). (A) positive control (gDNA IBET Gardel); (B) 1dpi; (C) 2dpi; (D) 3dpi; (E) 4dpi. M: Molecular marker. N: Negative control.

Fig. 3.7

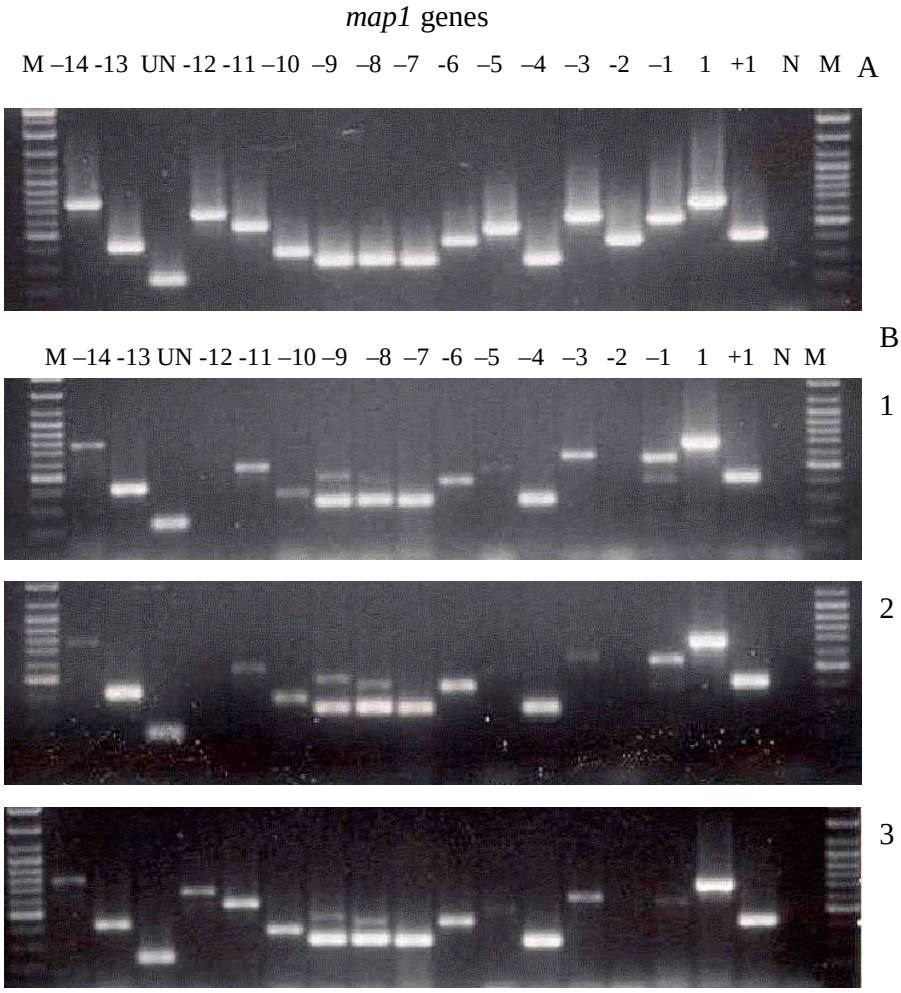


Fig. 3.7: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in endothelial cells (BUE) after 3 dpi. (A) positive control (gDNA IBET Gardel); (B) cDNA in triplicates (B1, B2, B3) for each gene. N: Negative control. M: Molecular marker.

Fig. 3.8

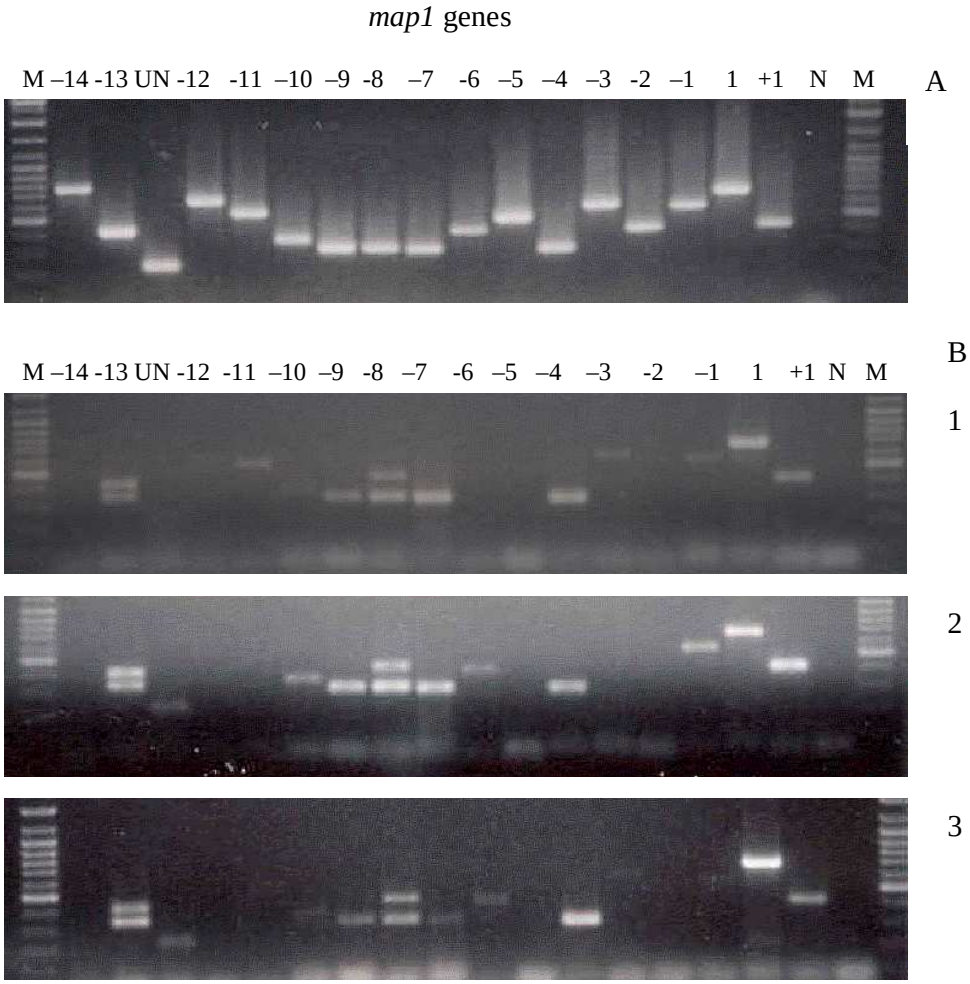


Fig. 3.8: Transcription of the *map1* cluster of *E. ruminantium* (CTVM Gardel) in endothelial cells (BUE) after 6dpi. (A) positive control (gDNA IBET Gardel); (B) 6 dpi (B1, B2, B3) for each gene. N: Negative control. M: Molecular marker.

Table 3.1 Effects of day of infection and temperature on transcription of the *E. ruminantium map1* genes in bovine endothelial cell cultures

Cell line [a]	1	+1	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11	-12	UN	-13	-14
BPC 31°C	++	++	++			++		++	+	++	+	+	++			++	
BPC 37°C	+++	+++	+++		+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
BUE D1 p.i.	+++	+++	++		++	+++	+	++	+++	+++	+++	+	++	+	+	++	+
BUE D2 p.i.	+++	+++	++		++	+++	+	++	+	+++	+++	++	++	+	++	++	+
BUE D3 p.i.	+++	+++	++			++			+++	+++	+++	+++	+++	++	++	++	+
BUE D4 p.i.	+++	+++	++		+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	
BUE D3 1-3 p.i.	+++	+++	++		++	+++	+	++	+++	+++	+++	++	++	++	++	++	+
BUE D6 5-7 p.i.	+++	++	+		+	++		+	++	++	++	++	++		++	++	

[a] *E. ruminantium* Gardel (CTVM)-infected bovine pulmonary artery (BPC) or umbilical cord (BUE) endothelial cell cultures were incubated at 31°C and 37°C. Symbols indicate: weak (+), clear (++) or strong (+++) signals.

Table 3.2 Effects of type of tick cell line and temperature on transcription of *map1* genes

	Map1 paralog numbers																
Cell line [a]	1	+1	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11	-12	UN	-13	-14
AVL13 31°C	+++	++	+++			+			+	+	+	+	+		+	+	+
AVL13 37°C	++	++	+++						+	+					+	+	+
RAN3 31°C	+	+	++														
RAN3 37°C			+														
IDE8 31°C	++	+	+++			+			+	+	+		+		+	+	+
IDE8 37°C			+														

[a] *E. ruminantium* Gardel (CTVM)-infected cell lines *I. scapularis* (IDE8), *A. variegatum* (AVL/CTVM13) and *R. appendiculatus* (RAN/CTVM3) were incubated at 31°C and 37°C. Symbols indicate: weak (+), clear (++) or strong (+++) signals.

3.4 Discussion

One of the main objectives of the experiments reported here was to determine whether transcription of the *map1* multigene family is influenced by temperature, by studying *E. ruminantium*-infected endothelial and tick cell lines incubated at two different temperatures, 31°C and 37°C. The higher temperature, 37ºC, serves as a temperature model for ruminants; 31ºC was chosen as approximately the lowest temperature at which the cells could be maintained.

Overall, the results of these experiments confirmed that differences exist in the transcription of the *map1* multigene family in host and vector cell environments, which is in line with previous results published by van Heerden et al. (2004) and Bekker et al. (2005). However, in the previous studies all *map1* paralogs were found to be transcribed in *E. ruminantium*-infected endothelial cell cultures regardless of sources of cells used (bovine pulmonary artery BPC, umbilical cord BUE or aorta endothelial BA and BAE), the bacterial isolate used (Gardel CTVM, Gardel IBET, Welgevonden and Senegal) or the temperature at which cultures were maintained (30°C and 37°C). The results of the present study indicate that, while all *map1* paralogs are transcribed when *E. ruminantium* is grown in BPC maintained at 37ºC, a number of the genes are apparently not transcribed in cultures maintained at 31ºC. Thus, no PCR products were detected for paralogs *1-3, 1-5, 1-12, UN and 1-14*. Similar discrepancies have been reported in transcriptional studies of the multigene families of *E. canis* and *E. chaffeensis*. McBride et al. (2000a) reported that five *p28* genes of *E. canis* were transcriptionally active in infected DH82 cells at the vertebrate host (37ºC) and ambient tick (27ºC) temperatures whereas Unver et al.

(2001) found that all 22 *p28* paralogs were downregulated or undetectable (with the exception of *p30-10*) when *E. canis* was cultivated in DH82 cells at 25ºC. Differences in the mRNA expression and documentation of multiple transcripts have been attributed to a number of possible causes, including the use of different primer pairs in different laboratories (Bekker et al., 2002; Reddy et al., 1998; McBride et al., 2000a; Yu et al., 2000). However, use of different primers would not explain discrepancies between the results of the present experiments and those of van Heerden et al. (2004) and Bekker et al. (2005) since the same RT-PCR methodology and gene-specific primers for each *map1* paralog were used in all three studies. Another possibility is that transcription may be influenced by different culture conditions and techniques (Ohashi et al., 2001; Unver et al., 2001; Long et al., 2002; Cheng et al., 2003). The different pattern of transcription in the cultures at 31ºC in comparison with previous data from cultures at the same temperature (van Heerden et al., 2004; Bekker et al., 2005) could thus be attributed to the use of different culture techniques and/or to the use of different batches of RNA made from cultures harvested at different time points in different laboratories, as has been also suggested by Cheng et al. (2003). The possibility cannot be excluded that in the cultures used for this study, infected cells maintained at sub-optimal temperatures could have been functionally affected and therefore the transcriptional activity of the bacteria decreased. In addition, the observed difference in the amount of bacteria in cultures grown at 31°C and 37°C could account for some of the differences observed in transcription. Lower proportions of bacterial message in the RNA samples could result in reduced sensitivity for detection of those paralogs transcribed at low levels.

This might be overcome by using quantitative PCR and including measurement of transcription of bacterial housekeeping genes.

Regarding analysis of *map1* transcripts in *E. ruminantium*-infected tick cells, only small differences were observed in the pattern of transcription between infected AVL/CTVM13 at 31°C and 37°C. Transcripts for two genes, *map1-4 and 1-10* were detected in cultures grown at 31ºC (very faint band) but not detected in cultures grown at 37ºC. AVL/CTVM13 can be maintained at 31°C and 37°C, and has been successfully infected with *E. ruminantium* (Gardel) at both temperatures. However, attempts to adapt RAN/CTVM3 and IDE8 to grow continuously at 37°C have been unsuccessful and therefore these cultures were adjusted to 37°C only one week before harvesting the cells. Bacteria in these cultures were found to be transcribing ribosomal genes, although at lower levels for RAN/CTVM3 (Fig. 3.1), indicating that viable *E. ruminantium* are still present after 1 week of incubation at 37ºC; however, the transcription of the *map1* cluster at 37°C in RAN3 and IDE8 was greatly reduced or undetectable. The reduced level of bacteria and diminished transcription of *map1* paralogs -and also of 16S ribosomal genes- in *E. ruminantium*-infected cultures maintained at 37°C in comparison with those maintained at 31°C suggest that growth of these cells at 37°C is not conducive for multiplication of the organisms. Consequently, results obtained from comparison of organisms grown in these cultures at different temperatures are difficult to interpret.

Nevertheless, it was possible to compare the results of transcription in AVL/CTVM13 at 37°C and IDE8 and RAN/CTVM3 at 31°C with those reported by Bekker et al (2005) in the same tick cell lines, infected with the same isolate (CTVM Gardel) and maintained at the same temperatures. The pattern of transcription in

AVL/CTVM13 at 37ºC was nearly identical to that reported by Bekker et al. (2005), with the exception that transcripts for two genes detected by Bekker et al. (2005), *map1-4 and 1-9*, were not detected in the present experiments. The pattern of transcription in IDE8 at 31ºC in the present study was also very similar to that reported previously (Bekker et al 2005), but three genes *map1-11, UN, 1-14* were found to be transcribed in this study, albeit at lower levels, and not detected in the previous study. In RAN/CTVM3 at 31°C transcription of *map1* paralogs was even more diminished, with only transcripts for three genes detected. Similarly Bekker et al (2005) reported transcription of only five genes in Gardel-infected RAN/CTVM3 at 31°C, two of which (*map1+1* and *map1-1*) were also transcribed in the present study. These differences may be due to small differences in sensitivity in individual assays due to different batches of reagents.

The results of these experiments do not provide a clear answer regarding the role, if any, of temperature in regulating the transcription of the *E. ruminantium map1* multigene family. Dramatic differences in transcription of *map1* paralogs were not observed between *E. ruminantium*-infected AVL/CTVM13 cultures grown at 31°C and 37°, although transcription at lower temperatures (e.g. 25°C), which are likely to represent the temperature in the tick vector, was not investigated. Levels of transcription in *E. ruminantium*-infected endothelial cell cultures did not appear to be be temperature-sensitive as a similar pattern of transcription was found in AVL/CTVM13 at 31°C and 37°C. It was not feasible to investigate transcription at temperatures below 30°C as such low temperatures restrict the growth of *E. ruminantium* in endothelial cells. The standard PCR used in this study is only likely to detect substantial differences in gene transcription. Real time PCR assays would

be required to provide more precise measures of transcript levels and thus to reveal quantitative differences in expression.

A second objective of this chapter was to investigate if differential transcription of the *map1* cluster exists between different developmental forms of *E. ruminantium*. A method for separation of reticulated bodies from elementary bodies in *E. ruminantium* was not available; therefore different fractions of cultures enriched for one or another developmental form were used (Jongejan et al., 1991c; Marcelino et al., 2005).

Evidence for differences in the pattern of transcription of the *map1* cluster between morphologically different forms of *E. ruminantium* was found. While all paralogs were found to be transcribed in intracellular forms of *E. ruminantium* at day 3 p.i. (Fig. 3.7), paralogs *map1-5, 1-12 and 1-14* were not detected in organisms harvested from supernatant of infected cultures at day 6 p.i. (Fig. 3.8). Furthermore, a general decrease in intensity of the signal of RT-PCR products was observed for all *map1* genes found to be transcribed on day 6 p.i. compared with samples from day 3 p.i. The decrease of transcription observed in elementary bodies might not be surprising as they are in a non-replicative stage and therefore are likely to be metabolically less active. Elementary bodies of *Chlamydia trachomatis* have been well characterised and are defined as particles with little or no metabolic activity and with a rigid outer membrane protein that enables organisms to attach to and enter host cells (Caldwell et al., 1981; Hatch et al., 1984). However, Belland et al. (2003) found EBs from *C. trachomatis* to be transcriptionally active as early as 1h after addition to mammalian cells, long before EBs had developed into RBs. It is possible that extracellular *E. ruminantium* in the supernatant medium are still sufficiently active to transcribe

some of the paralogs of the *map1* cluster. However, the possibility that samples from d6 contained some reticulate bodies, which were responsible for detection of *map1* paralogs, cannot be excluded.

If the pattern of expression of *map1* genes is related to infectivity for the mammalian host, it might be expected that the pattern of transcription of immature forms of *E. ruminantium* growing in endothelial cells would be similar to that found in *E. ruminantium* growing in tick cells. This notion would be consistent with the observation of reduced infectivity and/or absence of clinical response when animals are either inoculated with *E. ruminantium* grown in tick cell lines (Bell-Sakyi et al., 2002) or with immature forms of the organism present in endothelial cells (Jongejan et al, 1991c). However contrary to this line of thinking, it was found that the pattern of transcription from elementary bodies, the infectious form of *E. ruminantium*, and not that from reticulate bodies, was very similar to the pattern of transcription observed in infected tick cells. This fact suggests that the reduced infectivity of tick stages of *E. ruminantium* and immature forms of *E. ruminantium* in endothelial cells is not closely correlated with differential transcription of the *map1* cluster.

An additional interesting observation is that some *map1* paralog transcripts (*map1-3, 1-5, 1-6, 1-10, 1-12, and 1-14*) were either never detected or were substantially downregulated in the different tick cell lines in both the present study and that of Bekker et al. (2005), independent of temperature or RNA batch. This could be due to: (i) the presence of relatively weak promotors for these particular paralogs, as found by Barbet et al. (2005) in the genomic expression site of *A. phagocytophilum*; (ii) diminished transcription of these genes triggered by special conditions e.g. when a culture is biased to a specific developmental form in a specific host cell; or (iii) low

sensitivity of the RT-PCR when using the primers for these paralogs. No studies have been carried out so far to determine promotors for the *map1* multigene family. Brayton et al. (1997), working with an *E. ruminantium* expression library, suggested that promotors upstream of the *map1* gene are active in *E. coli* and that recombinant MAP1 protein could have been expressed from its own promoter. However, as the nucleotide sequence of *E. ruminantium* has a high A+T content (70%) numerous possible promoter sequences can be identified upstream of the *map1* gene (van Vliet et al, 1994). Recently it was shown by van Heerden et al. (2004) that the paralogs from *map1-12* to *map1-2* are all transcribed polycistronically (when *E. ruminantium* is grown in endothelial cells) and therefore it is likely that a single promoter might regulate all these paralogs. As we found that some paralogs are transcribed when the adjacent paralog is not, e.g. *map1-7* transcripts were always present in infected tick cells while *map1-6* transcripts were never detected, it is necessary to look for other alternative explanations. The possibility exists that additional regulatory sequences might occur between paralogs located in the polycistronic area (*map1-12* to *map1-2*), as intergenic regions as large as 42 bp have been reported. In this case, it might be possible that some paralogs are independently regulated under specific conditions. Further studies are needed to determine if host factors are involved in the downregulation of these paralogs. The low sensitivity of specific primers seems to be the most likely explanation for the constant detection of the same downregulated paralogs in tick cell cultures, as a similar pattern of transcription was also found in both Gardel-infected BPC at 31°C and late stage cultures (rich in EBs) of Gardel-infected BPC at 37°C. To resolve this uncertainty the sensitivity of the RT-PCR must be evaluated for all the downregulated genes. Unfortunately, it was not

possible to construct plasmids containing inserts of all these paralogs and measure the sensitivity of specific primers to amplify specific paralog transcripts. Evaluation of the sensitivity of the RT-PCR is strongly recommended for future studies.

In summary, a different pattern of transcription of the *map1* cluster between different forms of *E. ruminantium* was found. However, no obvious correlation was found between this differential pattern of transcription and infectivity of *E. ruminantium*, as a similar pattern was found in elementary bodies, the infective form of the bacteria in the mammalian host (Jongejan et al., 1991c), and *E. ruminantium* tick cell culture stages, which do not normally cause disease when inoculated into the mammalian host (Bell-Sakyi et al., 2002). Further analysis should be carried out on synchronised cultures and ideally be accompanied by electron-microscopy studies and purification of the different developmental forms of the bacteria.

In addition it is not clear whether the findings described here are the result of asynchronous transcription of different individual genes by different *E. ruminantium* organisms, or of simultaneous gene transcription by all the organisms from an *in vitro* culture. Nor is it clear that the *in vitro* findings accurately reflect what is happening *in vivo* in the respective hosts. Therefore it is necessary to translate such studies to the *in vivo* situation and investigate the expression of the *map1* multigene family in the tick vector and the mammalian host.

Chapter 4: Transcriptional activity of the *map1* multigene family in midguts and salivary glands of *E. ruminantium*-infected ticks

4.1 Introduction

Differences in transcription of the *E. ruminantium map1* multigene family *in vitro*, in host and vector cell environments, have been reported (van Heerden et al., 2004; Bekker et al., 2005) and described in Chapter 3 of this thesis. In the related pathogens *E. canis* and *E. chaffeensis*, differences in transcription of multigene families encoding outer membrane proteins have been found between vectors and mammalian hosts, not only *in vitro* but also *in vivo*, and are suspected to have a role in tick transmission and adaptation to different hosts of ehrlichial pathogens (Ohashi et al., 1998b; Reddy et al., 1998; Yu et al., 1999, 2000).

The aim of the experiments described in this chapter was to analyse the transcriptional activity of the *map1* cluster in different tissues of infected ticks. Understanding the role that these genes may play in the life cycle of *E. ruminantium* in ticks might provide clues regarding survival of this pathogen in the tick vector and efficient transmission from the tick vector to the mammalian host.

Accordingly, transmission experiments were carried out involving acquisition feeding of larval or nymphal *A. variegatum* ticks on naïve sheep experimentally infected with *E. ruminantium* in the form of *in vitro* cultivated elementary body or blood stabilates, and subsequent transmission feeding of the resultant potentially infected nymphs or adult ticks on further naïve sheep. Molecular methods (real-time PCR and RT-PCR) were evaluated using *in vitro*-derived bacteria and plasmids, and used to detect and provide relative quantitative measures of *E. ruminantium* organisms in nymphal and adult ticks before and during transmission feeding, and to analyse transcription of the *map1* multigene family in these ticks.

Further validation of the molecular methods used in this chapter was performed in order to confirm that the sensitivity and specificity of the PCRs and RT-PCRs were adequate to detect *E. ruminantium* in experimentally infected ticks.

4.2 Materials and Methods

4.2.1. Growth and harvest of *E. ruminantium* in bovine endothelial cells and tick cell lines.

The CTVM subpopulation of the Gardel isolate of *E. ruminantium* (Uilenberg et al., 1985; Bekker et al., 2005) was cultured in bovine pulmonary artery (BPC) or bovine umbilical endothelial (BUE) cells and *A. variegatum* (AVL/CTVM13) tick cells at 37ºC and 31ºC respectively as described previously (Mutunga et al., 1998; Bell-Sakyi et al., 2000a). Bacterial growth was monitored in Giemsa-stained cytocentrifuge smears. DNA and RNA were extracted from both infected and uninfected cultures (section 4.2.5) to be used as positive and negative controls respectively.

4.2.2. Laboratory propagation of ticks. *A. variegatum* ticks were kindly provided by Dr Dominique Martinez of the Centre International de Recherche Agronomique pour le Développement (CIRAD-EMVT), Guadeloupe, and established as an *E. ruminantium*-free laboratory colony at Utrecht University. Larvae and nymphs were fed on rabbits, while adults were fed on sheep according to the method of Heyne, Elliot & Bezuidenhout (1987). The engorged, moulting and unfed stages were incubated at 27ºC with a relative humidity of 95%.

4.2.3. Experimental infection of sheep and ticks. Maintenance of sheep and stabilates used for their infection are described in detail in Chapter 2, sections 2.2.2. and 2.2.2.2. Advice on the appropriate number of ticks of each instar to be

applied to the sheep was kindly provided by Dr Alan Walker (CTVM, University of Edinburgh) and Dr Cornelis Bekker (Utrecht University). Larvae were fed on the experimental sheep in cloth bags applied on both ears, while nymphs and adults were fed in cloth patches applied to the back of the animal. Table 4.1 shows all sheep numbers and tick stages used in these transmission experiments.

4.2.3.1. Transmission experiment 1: Sheep 3148 and 3175 were inoculated on day 0 with 1 ml of a 1:10 dilution in GMEM of STAB1; a third sheep 3183, was retained as an uninfected control. Approximately 200 uninfected *A. variegatum* nymphs were applied in cloth patches on the back of each sheep on days 5, 6, and 7 of infection and allowed to feed during the period coinciding with the febrile reactions of the infected sheep (Annexes 3 and 4). Engorged nymphs were collected and incubated for 6 weeks to allow for moulting and hardening. The resultant male and female ticks from sheep 3148 (3148M, 3148F), 3175 (3175M, 3175F) and from the control 3183 (3183M, 3183F) will be hereafter referred to using these codes (Table 4.1). To examine transmission of infection by these adult ticks, first five uninfected male ticks were placed in a body patch on each of three new *Ehrlichia*-free sheep (3180, 3149, 3190) to allow release of pheromones to enhance the subsequent attachment of females. Five days later, 30 uninfected females (3183F) or supposedly infected female ticks (3148F and 3175F) were applied in the same patch as the colony males on sheep numbers 3180, 3149 and 3190 respectively. In addition, a second patch was used to apply 10 supposedly infected males, 3148M and 3175M, to sheep 3149 and 3190 respectively. Following attachment, five supposedly infected females and two supposedly infected males were detached daily on days 1-5 of feeding from each

test sheep (3149 and 3190) and five uninfected females were detached daily from the control sheep (3180). Midguts and salivary glands were dissected and pooled from unfed ticks (from the same original batches) and from feeding ticks immediately after detachment. DNA, RNA and proteins were extracted from midguts and salivary glands of each tick pool using the Tri reagent protocol.

4.2.3.2. Transmission experiment 2: Sheep 3154 was inoculated with 1 ml of a 1:10 dilution of STAB1 as before. Approximately 200 *A. variegatum* larvae were applied on each of days 5, 6, and 7 on test sheep 3154 and allowed to feed during the febrile reaction of the test animal. Engorged larvae were collected and incubated for 4 weeks to allow for moulting and hardening. Three hundred of the resultant moulted nymphs that had fed on the infected sheep 3154 (3154N) were placed on the back of a second susceptible sheep 3471. Following attachment, 60 nymphs were detached on each of days 1, 3 and 5 of feeding. Pools of 30 unfed and feeding nymphs from each day were immediately submitted to DNA and RNA extraction. Sheep 3471 was challenged with 2 ml of stabilate CR366, consisting of *E. ruminantium* (Gardel isolate) infected blood, 1.5 months after the tick application.

4.2.3.3. Transmission experiment 3: Sheep 3464 was inoculated on day 0 with 2 ml of stabilate CR366 consisting of *E. ruminantium* (Gardel isolate) infected blood. Approximately 150 uninfected *A. variegatum* nymphs were applied on each of days 7, 8, 9 and 10 after inoculation and allowed to feed during the febrile reaction of the animal. Engorged nymphs were collected and incubated for 6 weeks to allow for moulting and hardening. Of the resultant adults, 48 females (3464F) and 37 males

(3464M) were fed on the back of a second susceptible sheep 3456. Following attachment, five females and three males were detached daily on days 1-5 of feeding. Midguts and salivary glands were dissected and individual tissues pooled from unfed females and from feeding females immediately after detachment from animal 3456. The pool of intact males and the pooled tick tissues from females were submitted to DNA and RNA extraction.

4.2.4. Enzyme-linked immunosorbent assay (ELISA). In order to check seroconversion in infected sheep, the indirect ELISA for detection of antibodies to recombinant MAP1-B antigen of *E. ruminantium* (van Vliet et al., 1995) was carried out. The MAP1-B indirect ELISA was supplied in kit form by Utrecht University and the assay was carried out as previously described (Mboloi et al., 1999). Briefly, MAP1-B antigen was diluted (1.4 µg/ml) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) and immobilised onto 96-well ELISA plates (Microlon Multibind immunoassay plates; Greiner Labortechnik) by incubation for 1 h at 37°C and then stored overnight at 4°C. One hundred microliters per well was used in all the steps described below. Plates were incubated for 15 min at 37°C with PBS-TM blocking buffer (phosphate-buffered saline [PBS], pH 7.3, supplemented with 0.1% Tween 20 and 1% dry skimmed milk [ELK Campina, Eindhoven, The Netherlands]). Plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and subsequently incubated with sera diluted (1:200) in PBS-TM for 1 h at 37°C. Plates were washed three times with PBS-T and incubated for 1 h at 37°C with rabbit anti-sheep antibodies conjugated with horseradish peroxidase (RαSh/IgG[H+L]/PO; Nordic) diluted (1:2000) in PBS-TM. ELISA plates were washed three times with PBS-T, and freshly prepared ABTS [2,2'-azinobis (3-ethylbenzthiazoline sulphonic

acid)] substrate was added. Colour development was allowed for 30 min in the dark, and absorbance was measured at 405 nm with an ELISA reader (Ceres UV 900 C; Biotek Instruments BV, Abcoude, The Netherlands). Each plate included one positive and one negative control serum sample (provided with the kit) and all samples were analyzed in duplicate on the same plate. The means of the duplicate optical density (OD) measurements were calculated for each sample, and the results were expressed as the percent positivity (PP) calculated as a percentage of the mean OD value of the reference positive control.

4.2.5. DNA and RNA isolation. Pooled adult tick midguts and salivary glands or pools of intact nymphs or males, were frozen in liquid nitrogen and ground into powder. Simultaneous DNA and RNA extraction from the resultant homogenised tick tissues was performed using the TRI reagent protocol (Sigma) according to the manufacturer's instructions. After extraction, samples were "cleaned" using the columns from Qiagen DNA and RNA extraction kits. Total DNA and RNA from *E. ruminantium*-infected and control uninfected endothelial and tick cell cultures were extracted using QIAamp (Qiagen) extraction kits according to the manufacturer's instructions.

4.2.6. cDNA synthesis and paralog-specific PCR. Ten microliters of DNase-treated RNA was used to prepare 40 µl of cDNA using 100 ng of random hexamer primers of the SuperScript^{TM} first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. When needed, cDNA was also prepared by using 2µM of MAP1-1R specific primer. Control samples were prepared by omitting reverse transcriptase from the reaction. Two microliters of cDNA was

subsequently used as template in a PCR reaction containing: 1X Taq PCR buffer (Promega), 3 mM $MgCl_2$, 1.25 U of Taq polymerase (Promega), 400 µM of each deoxynucleoside triphosphate, 10 pmol of each primer in a 25 µl reaction. The sequences of the paralog-specific primers used are given in Table 1 of van Heerden et al (2004). Reactions were carried out on a iCycler (Bio-Rad Laboratories) using the following programme: 2 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C and a final elongation step of 7 min at 72°C. Positive controls incorporated genomic DNA instead of cDNA. PCR products were visualized by running the samples on agarose gels stained with ethidium bromide. When needed, sequencing of amplicons was carried out at Base Clear, The Netherlands.

4.2.7. Estimation of PCR sensitivity. The sensitivity of the PCR was estimated by amplifying samples with a known concentration of plasmid DNA containing the *map1-1* gene. Two different plasmid constructs containing the *map1-1* gene (plasmids: pGEM-Teasy, Promega; and pBAD/Myc-His, Invitrogen) referred to as MAP1-1A and MAP1-1B respectively, were used as a source of DNA to amplify fragments of 474bp or 182bp of the *map1-1* gene using MAP1-1F/R and F3/R1 primers respectively. The DNA concentration of the plasmid samples was determined by spectrophotometry as described before in Chapter 2 (section 2.2.5). Ten-fold dilutions (each series in duplicate for each plasmid) were then prepared from known concentrations of plasmid copies in milliQ water. One microliter from each dilution was submitted to PCR amplification under the same conditions as those described in Chapter 2 (sections 2.2.4 and 2.2.5). PCR products were visualized by running the samples on agarose gels stained with ethidium bromide.

4.2.8. Estimation of RT-PCR sensitivity. The sensitivity of the RT-PCR was estimated by creating *map1-1* transcripts *in vitro*, using the kit Riboprobe system-T7 (Promega) according to the manufacturer's instructions. Briefly, pGEM-Teasy vector MAP1-1A, which includes a T7 promotor and *map1-1* gene insert, was linearised with NaeI (New England Biolabs). The linearised plasmid was precipitated with phenol:chloroform:isoamyl alcohol and ethanol and resuspended in milliQ water before the production of "run-off" transcripts. For the synthesis of *in vitro* transcripts, the linearised plasmid was included in a 20µl reaction mixture as follows: 1X transcription buffer, 10mM DTT, 20 U of RNases inhibitor, 2.5mM of deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP) and 20 U of T7 RNA polymerase. The mixture was incubated at 37 ºC for 1 hour and removal of template DNA was then carried out by incubation of the sample at 37 ºC for 15 min with RNase-Free DNase (included in the kit). RNA transcripts were precipitated as before and resuspended in milliQ water. The concentration of the transcripts was determined by spectrophotometry and the number of single strand RNAs was calculated using the following formula:

X g/µl RNA / [transcript length in nucleotides x 340]) x 6.022×10^{23} = Y molecules/µl

cDNAs were synthesised as described above from transcripts diluted ten-fold (and a negative control with no reverse transcriptase). 2 µl of the first strands were used in a semi-nested PCR, with the same conditions as described above for the RT-PCR, using MAP1-1F/R primers for the first round and F3/MAP1-1R for the second round of PCR. PCR products were visualized in ethidium bromide-stained agarose gels.

4.2.9. PCR and semi-nested PCR on ticks for detection of *E. ruminantium*. For detection of *E. ruminantium* in whole ticks, tick tissues, infected blood or infected and uninfected tick cultures, several combinations of primers were used, always using the same PCR conditions as described above for RT-PCR (section 4.2.6). PCR products were visualized in ethidium bromide-stained agarose gels. Table 2.3 shows primer sequences and Fig. 4.1 shows the expected size of amplicons and DNA targets used in this chapter.

4.2.10. Real-time PCR on tick tissues. The *map1-1* gene of *E. ruminantium* was selected as the target to quantify bacterial DNA in midguts and salivary glands. MAP1-1B plasmids (10-fold dilution standards) and *E. ruminantium*-infected tick samples were submitted to PCR amplification to generate a fragment of 182 bp, using forward primer F3 and reverse primer R1 (Table 2.3) in a 25 µl reaction mixture, under the same conditions as those described in Chapter 2 (section 2.2.6.2). Ct values were determined and melting curve graphs analysed accordingly using the iCycler software.

Table 4.1: Acquisition and transmission feeding of *A. variegatum* ticks[b]

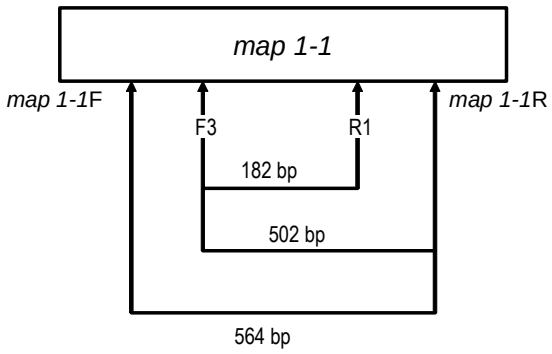
	Acquisition feeding				
	Sheep[a]	Stabilate	Tick stage applied	Code of resultant ticks	Sheep used for transmission feeding[c]
Experiment 1	3148	STAB1	Nymphs	3148F 3148M	3149
	3175	STAB1	Nymphs	3175F 3175M	3190
	3183	Control	Nymphs	3183F 3183M	3180
Experiment 2	3154	STAB1	Larvae	3154N	3471
Experiment 3	3464	CR 366	Nymphs	3464F 3464M	3456

[a] Sheep N^{o} 3148, 3175, 3154 and 3464 were inoculated with *E. ruminantium* stabilates as indicated. Sheep N^{o} 3183 was used as a control.

[b] Nymphs were allowed to feed on all but sheep N^{o} 3154, on which larvae were applied. After engorgement and moulting, each batch of ticks was identified by the number of the donor sheep plus F (female) or M (male), or N (nymph) for those fed on sheep 3154.

[c] Moulted and hardened ticks, resulting from acquisition feeding, were applied on susceptible sheep for transmission feeding.

(A) Detection of *E. ruminantium* DNA/cDNA in tick samples



(B) Detection of *map1-1 in vitro* transcripts

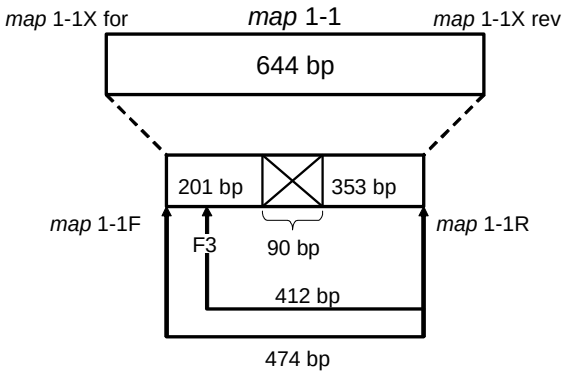


Fig. 4.1: The *map1-1* gene (A) or plasmid encoding the *map1-1* gene (B) was targeted with F3/R1 or *map1-1* F/R primers to render specific fragments during single or nested PCRs for detection of *E. ruminantium* in whole ticks, tick tissues, infected blood or infected and uninfected tick cultures. Arrows indicate putative positions on the gene. Sizes of amplicons generated are expressed in base pairs (bp).

4.3 Results

4.3.1. Validation of the Tri reagent protocol for extraction of DNA, RNA and protein.

4.3.1.1. Whole ticks: In order to determine whether *E. ruminantium* DNA, RNA and proteins could be isolated efficiently from the same sample, pools of 4 uninfected and 4 *E. ruminantium* (Senegal)-infected unfed female *A. variegatum* ticks, were submitted to extraction according to the Tri reagent protocol. The 16S ribosomal gene of *E. ruminantium* was used as a target for amplification by both PCR and RT-PCR. Amplification from 1, 3 and 4 µl samples of DNA suspension from infected ticks, using the 16S ribosomal primers, gave a band of the expected size (approx. 500 bp) (Fig. 4.2). A band of the same size was obtained from 5 and 7µl samples of DNA-clean RNA from infected ticks and the *E. ruminantium* positive control, using the same 16S ribosomal primers. Negative controls, without reverse transcriptase or DNA, did not give any amplification (Fig. 4.3). The protein fraction extracted from the same sample was subjected to electrophoresis in a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Clearly visible protein bands were present in gels stained with the Direct blue (DB70) staining method and differences were noted between infected and uninfected tick protein extracts (Fig. 4.4). No further effort was made at this point to investigate expression of *E. ruminantium* proteins.

4.3.1.2. Dissected tick tissues: To check the efficiency of the Tri reagent protocol for DNA and RNA extraction from dissected tissues, a pool of 4 *E. ruminantium* (Senegal isolate)-infected unfed female *A. variegatum* were dissected and their midguts and salivary glands each pooled and processed according to the protocol. The extracted DNA and DNA-clean RNA from *E. ruminantium*-infected midguts and salivary glands were submitted to PCR and RT-PCR. The *E. ruminantium*-derived endothelial cell cultures used as a positive control, genomic DNA from infected midguts and cDNA from midguts and salivary glands all gave a positive signal when amplified with the 16S ribosomal primers. Amplification of negative control DNA and genomic DNA from infected salivary glands did not give any products (Fig. 4.5A). However, when paralog-specific (*map1*, *map1-1*, *map1+1*) primers were used, amplification was only observed in the *E. ruminantium* positive control. The same cDNA samples prepared from infected midguts and salivary glands that previously gave a band when amplified with 16S ribosomal primers, did not give any amplification when paralog-specific primers were used (Fig. 4.5B). This result showed the need for the evaluation of sensitivity of the *map1* paralog-specific primers. In addition, the specificity of the *E. ruminantium* 16S and *map1* paralog-specific primers was evaluated.

Fig. 4.2

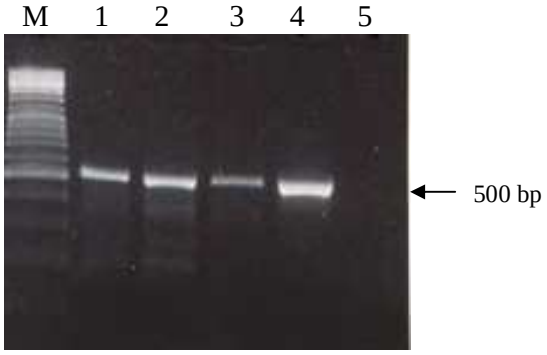


Fig. 4.2: PCR amplification with specific 16S ribosomal primers of *E. ruminantium* in ticks infected with the Senegal strain, using 1µl (lane 1), 3 µl (lane 2) and 4 µl (lane 3) of DNA extracted with Tri reagent from a pool of 4 infected ticks or from a positive control of *E. ruminantium*-derived endothelial cells (lane 4). Lane 5: no template control. M: molecular marker.

Fig. 4.3

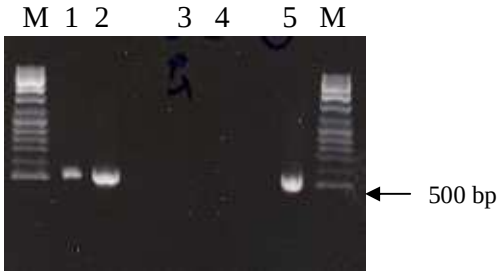


Fig. 4.3: Transcription of the 16sRNA gene in infected ticks (Senegal isolate) using 5 µl (lane 1) and 7 µl (lane 2) of RNA extract. Lane 3: no template control; lane 4: negative reverse transcriptase control; lane 5: positive control (*E. ruminantium* gDNA). M: molecular marker.

Fig. 4.4

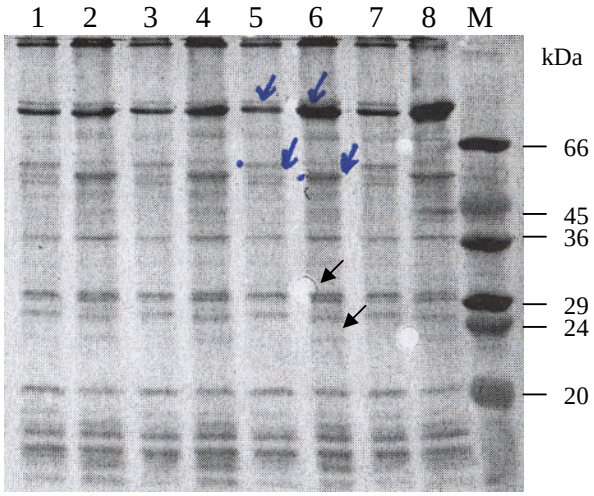


Fig. 4.4: Comparison of 12.5 % SDS-PAGE pattern of proteins from *E. ruminantium* infected (lanes 2, 4, 6, 8) and uninfected (lanes 1, 3, 5, 7) ticks extracted using the Tri reagent protocol. Arrows indicate bands that are present or over-expressed in lanes containing *E. ruminantium* infected tick extracts but absent in the uninfected tick extracts. M: SDS-7 molecular marker.

Fig. 4.5

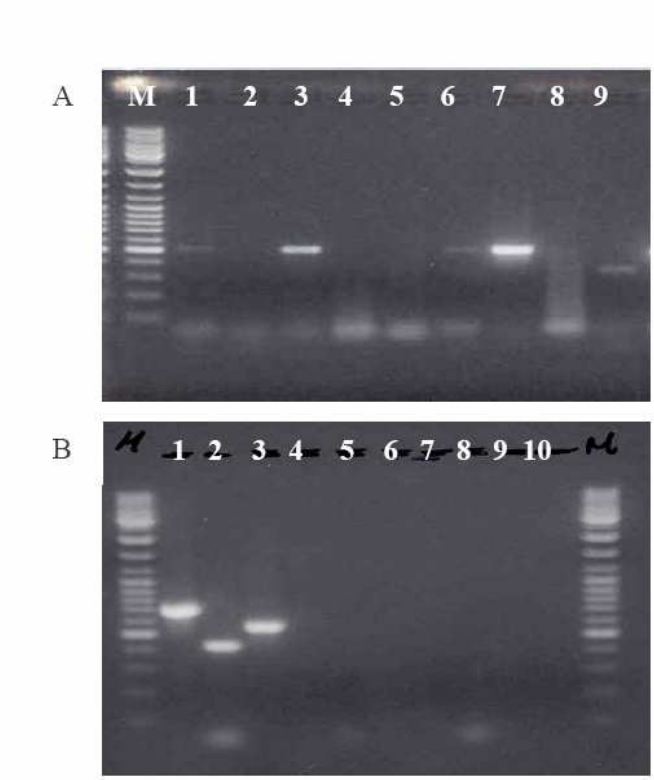


Fig. 4.5: Transcription of the 16S rRNA gene (A) and *map1*, *map1+1* and *map1-1* genes (B) in ticks. (A) Lane 1: cDNA from salivary glands (sg). Lanes 2 and 4: negative reverse transcriptase controls. Lane 3: cDNA from mg. Lane 5: gDNA from sg. Lane 6: gDNA from mg. Lane 7: *E. ruminantium* gDNA positive control. Lane 8: No template control. Lane 9: positive RT-PCR control. M: Molecular marker. (B) Lanes 1, 2, 3: gDNA positive control; lanes 4, 5, 6: *map1*, *map1+1* and *map1-1* cDNA in sg; lane 7, 8, 9: *map1*, *map1+1* and *map1-1* cDNA in mg. Lane 10: no template negative PCR control. M: Molecular marker.

4.3.2. Evaluation of the specificity of tick-specific Cyt C, *E. ruminantium* 16S ribosomal and *E. ruminantium map1* paralog-specific primers.

To assess primer specificity, genomic DNA from infected and uninfected ticks, and from uninfected tick cell cultures (AVL13/CTVM) was submitted to PCR using different combinations of primers. gDNA from infected ticks and the *E. ruminantium* positive control gave a signal of the expected size when DNA was amplified with primers for the *E. ruminantium* 16S ribosomal gene. DNA from uninfected and infected ticks and an uninfected tick cell line (AVL/CTVM13), but not the *E. ruminantium* positive control, gave a band of the expected size (550 bp) when PCR was carried out with primers specific for the tick Cyt C gene (kindly provided by Karine Delrouxe of CTVM) (Fig. 4.6). The specificity of the paralog-specific primers was evaluated by running a PCR with the 16 different combinations of *map1*-paralog primers on gDNA from uninfected ticks. Only one of these sets of primers (*map1-4)* yielded a product from uninfected tick DNA and the band (400 bp) was larger than the expected size (300 bp) (Fig. 4.7B). In contrast, a positive signal of the expected size was obtained for each primer combination when the PCR was run on the *E. ruminantium* positive control demonstrating the specificity of the primers in amplifying *E. ruminantium* DNA (Fig 4.7A).

Fig. 4.6

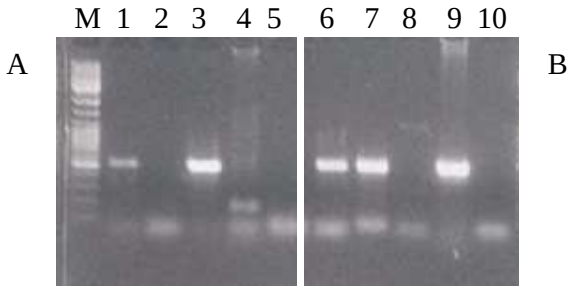


Fig. 4.6: PCR amplification of DNA from uninfected and infected ticks using ribosomal (A) and tick primers (B). Lanes 1, 6: gDNA from *E. ruminantium*-infected ticks. Lanes 2, 7: gDNA from uninfected (control) ticks. Lanes 3, 8: *E. ruminantium* positive control gDNA. Lanes 4, 9: positive control uninfected AVL/CTVM13 cells. Lanes 5, 10: no template PCR controls. M: molecular marker

Fig. 4.7

map1 genes

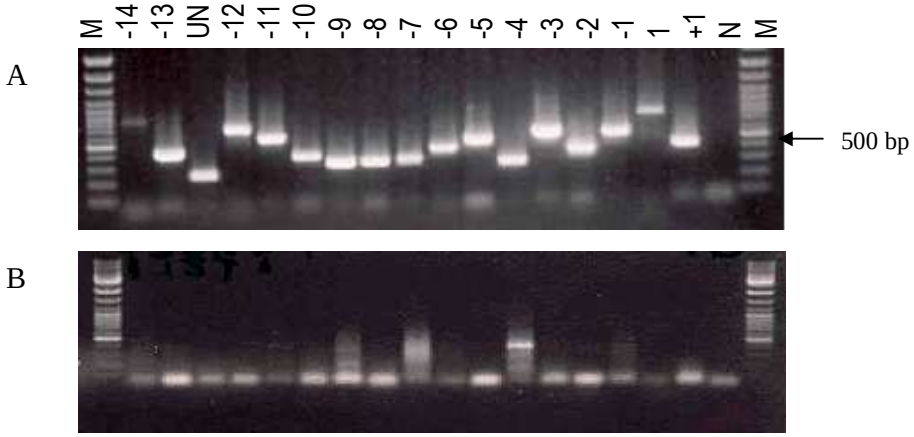


Fig. 4.7: Amplification of the *map1* cluster from *E. ruminantium* (Gardel)-infected endothelial cells (A) and uninfected ticks (B). N: negative PCR control. M: molecular marker.

4.3.3. Evaluation of the PCR sensitivity. The sensitivity of the PCR was evaluated by testing two different combinations of *map1-1* specific primers, in duplicate, to amplify 10-fold dilutions in milliQ water of a known concentration of plasmids MAP1-1A and MAP1-1B expressing the *map1-1* gene. Using MAP1-1 F/R primers a band of the expected size (474 bp) was observed in dilutions containing as few as 10 copies (Fig. 4.8A). A similar result was obtained with F3/R1 primers, giving a specific band of 182 bp; in one of the duplicates 10 copies were detected (Fig. 4.8B and Fig. 2.3 in Chapter 2).

4.3.4. Evaluation of the RT-PCR sensitivity. Since the *map1-1* gene is the paralog that is predominantly expressed in tick cell cultures (Bekker et al., 2005), the sensitivity of the RT-PCR in detecting *map1-1* transcripts was evaluated with MAP1-1F/R primers, the same combination of primers used for RT-PCR on tick tissues. *Map1-1* transcripts were synthesised *in vitro*, using the kit Riboprobe system-T7, and quantified by spectrophotometry as described above (section 4.2.8) and RT-PCR was carried out on 10-fold dilutions of these transcripts. In the first round of PCR, transcripts were detected in samples containing 1000 or more transcript copies using primers MAP1-1F/R. To increase the sensitivity of the system a second round of PCR was performed using F3/MAP1-1R primers in a semi-nested RT-PCR. The semi-nested RT-PCR allowed detection of transcripts in samples containing 10 or more copies (Fig. 4.9). Based on this result, the failure to detect transcripts of the *map1-1* gene (a single gene in the *E. ruminantium* genome (Collins et al., 2005)), in tick tissue samples would imply that either the samples contained very low numbers

of bacteria (less than 10) or that the bacteria were not actively transcribing the *map1-1* gene.

4.3.5. Evaluation of DNA and RNA loss after extraction. To determine the degree of DNA lost after extraction from ticks using the Tri reagent, a positive control sample of *E. ruminantium* gDNA was used to prepare a ten-fold dilution series from 10^{-1} to 10^{-6} in milliQ water. Artificially-infected tick samples were created by adding 1 µl of each gDNA dilution to 500 µl of Tri reagent containing uninfected ticks (previously ground into powder) or milliQ water prior to DNA extraction. This resulted in a final 100-fold dilution of the original *E. ruminantium* DNA sample used to artificially infect the Tri reagent sample. 1 µl of the extracted DNA from each of the resultant samples was submitted to amplification by PCR using the F3/R1 primers. A positive reaction was obtained from the control DNA sample in water down to a final dilution of 10^{-5} (10^{-6} gave a faint band). However, in the samples of the artificially-infected tick powder a positive reaction was obtained down to 10^{-4} indicating that there was a 10-fold loss of DNA during the extraction procedure (Fig. 4.10). In order to estimate the approximate number of organisms lost during the DNA extraction, further analysis was done by real-time PCR. A 10-fold dilution series were prepared using a known amount of *E. ruminantium* (Gardel) gDNA (calculated with reference to a standard curve generated by real time PCR) in Tri reagent sample containing uninfected tick powder. DNA was extracted from these samples into 100 µl of elution buffer and 1 µl samples were submitted to PCR amplification. Comparisons between the theoretical numbers of copies present in each sample versus the actual numbers of copies detected by real-time PCR indicated

a 10 fold reduction in the specific target DNA ($9x10^n$ organisms, n=3 to 7) during the extraction procedure (Fig. 4.11 and 4.12).

To assess whether *E. ruminantium* RNA could be extracted efficiently from tick tissues, a positive control sample of RNA (free of DNA) from *E. ruminantium*-derived endothelial cell cultures was used to prepare a ten-fold dilution series from 10^0 to 10^{-3} in water and five microlitres of each dilution were added to 500µl aliquots of Tri reagent containing uninfected tick tissues. RNA was extracted from these artificially-infected samples and cDNA synthesised using *map1-1*F/R primers. A signal was observed in the artificially-infected samples down to a dilution of 10^{-3} confirming the recovery of *E. ruminantium* RNA after extraction against a fixed concentration of host RNA without substantial loss of *E. ruminantium* RNA during the procedure (Fig. 4.13).

Fig. 4.8

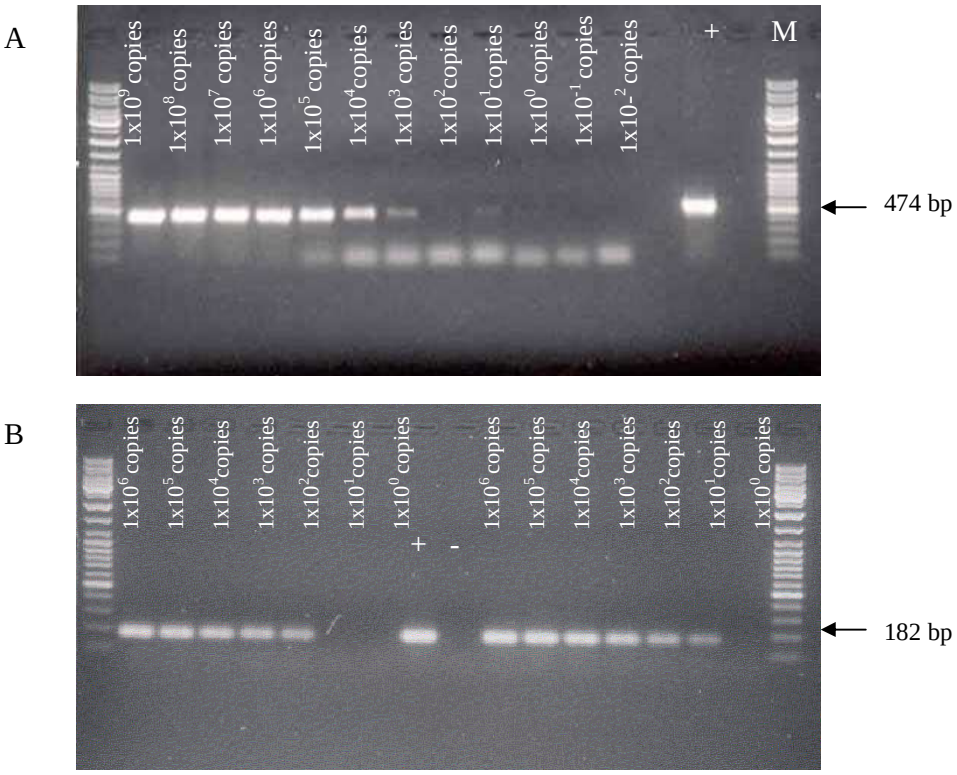


Fig. 4.8: Sensitivity of MAP1-1F/R (A) and F3/R1 (B) primers to amplify 10 fold-dilutions of, respectively, plasmids MAP1-1A and MAP1-1B, encoding the *map1-1*gene. +: *E. ruminantium* gDNA positive control. –: no template negative control. M: Molecular marker

Fig. 4.9

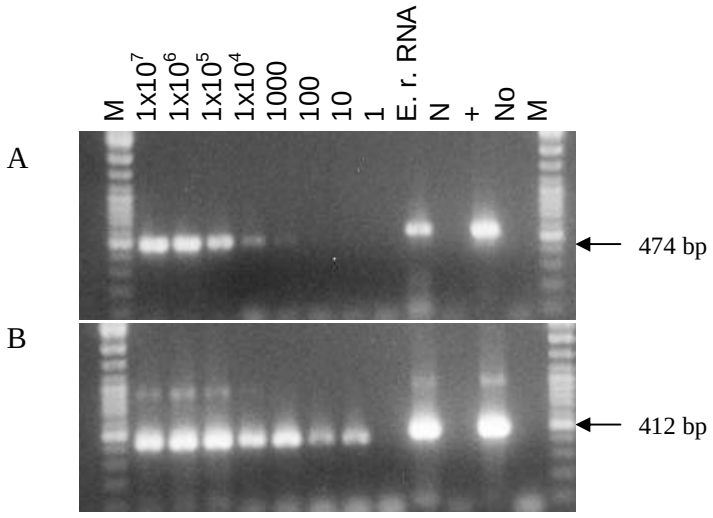


Fig. 4.9: Generation of specific cDNA from various concentrations (10-10^7 copies) of *in vitro*-synthesised *map1-1* transcripts using primers *map1-1* F/R (A) for the first round and F3/*map1-1*R (B) for the second round.

Fig. 4.10

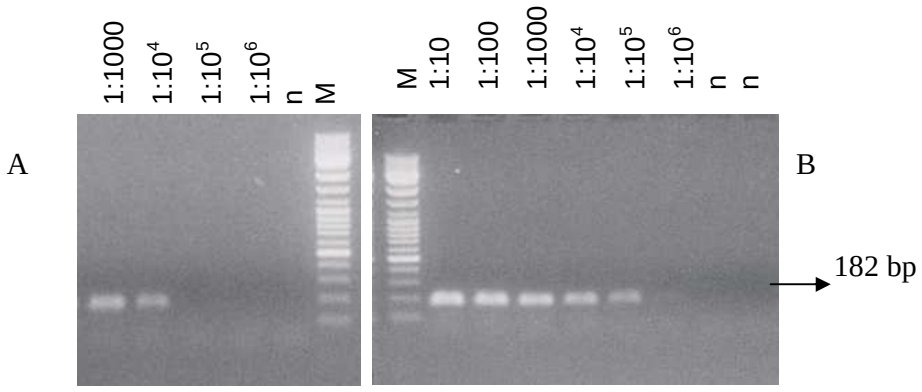
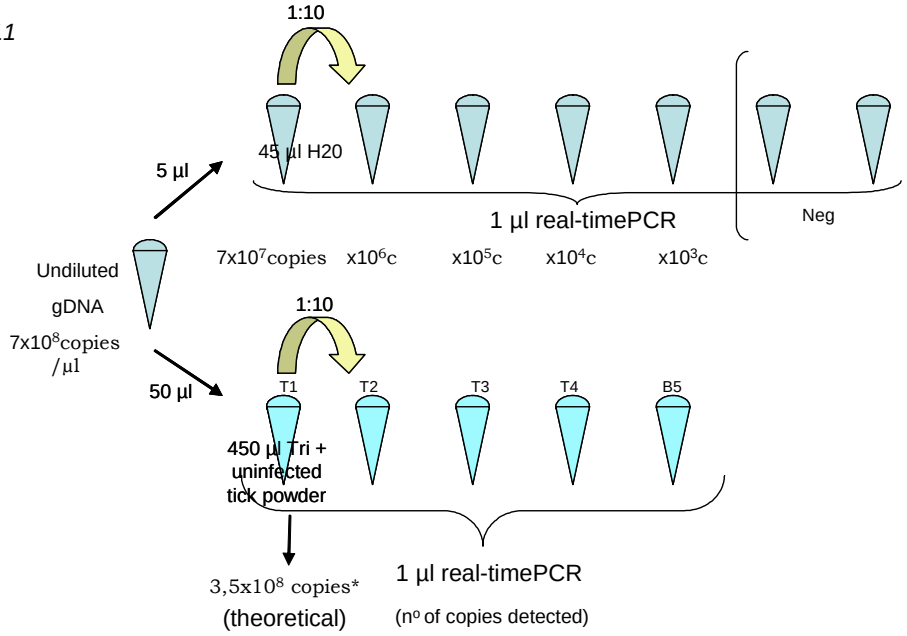


Fig. 4.10: Generation of PCR products from *E. ruminantium* gDNA diluted in uninfected tick tissues (A) or water (B) using primers F3/R1. n: negative control. M: molecular marker.

Fig. 4.11



* $7x10^8$copies-----1 µl

X --------50 µl

$X=3,5x10^{10}$

$3,5x10^{10}$ -------500 µl=100 µl DNA elution

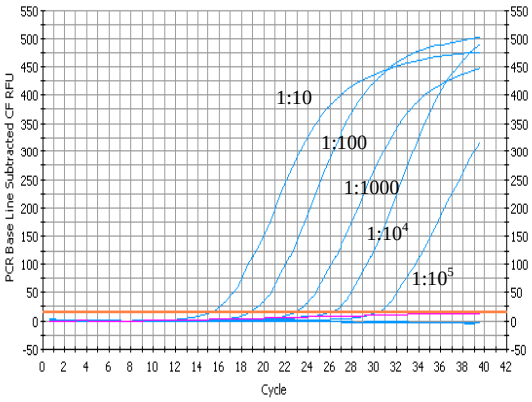
X----------1 µl DNA elution

$X=3,5x10^8$

Fig. 4.11 Diagrammatic representation of 10-fold dilutions of gDNA in water (A) and in uninfected snap frozen/crushed tick tissues (T1-T5) subjected to DNA extraction and real-time PCR in order to estimate gDNA loss. The theoretical number of genome copies in the first tick dilution sample (T1) was calculated (*) and compared to the number of copies quantified by the real-time PCR (Fig. 4.12).

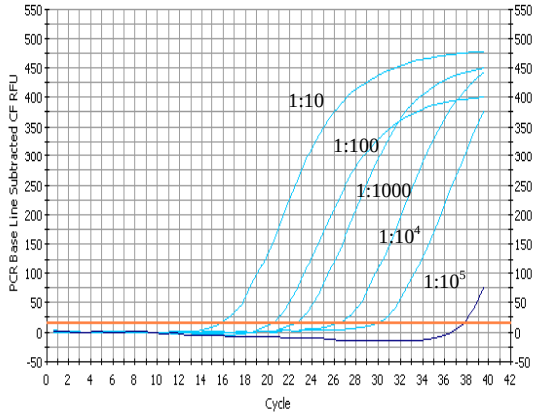
Fig. 4.12

A



gDNA dilutions	No of copies detected by real-time PCR in gDNA/water dilutions
1:10	$7x10^7$
1:100	$4,8x10^6$
1:1000	$2,8x10^5$
1:104	$2,7x10^4$
1:105	$1,28x\ 10^3$
1:106	Neg
1:107	Neg

B



gDNA dilutions in uninfected tick powder	Theoretical no of copies calculated in gDNA/blood dilutions if 100% recovery	No of copies in gDNA/blood dilutions detected by real-time PCR
T1	$3,5x10^8$	$7,6x10^7$
T2	$1x10^7$	$1,6x10^6$
T3	$1x10^6$	$3,4x10^5$
T4	$1x10^5$	$2,0x10^4$
T5	$1x10^4$	$1,67x10^3$
T6	-	-
T7	-	-

Fig. 4.12: Effects of dilution of gDNA in water (A) and in Tri reagent containing uninfected tick powder after DNA extraction (B) on the cycle threshold determined by real-time PCR. Quantities in corresponding tables express the number of copies of *E. ruminantium* in each dilution, calculated as in *Fig. 4.11* (theoretical) or detected by real-time PCR, per microlitre of DNA suspension.

Fig. 4.13

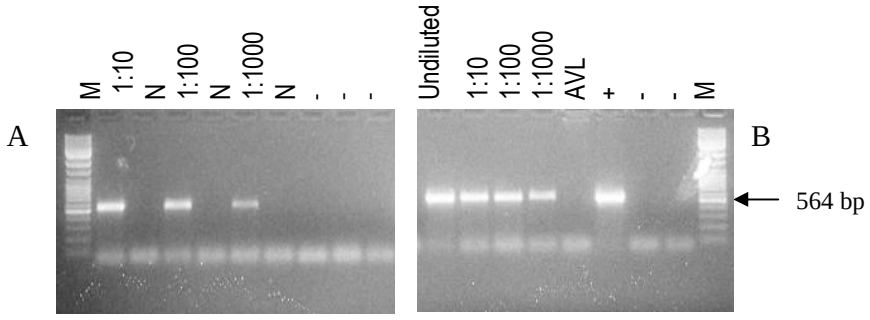


Fig. 4.13: Effects of dilution of *E. ruminantium* RNA in Tri reagent containing uninfected tick powder (A) or water (B) on detection of *map1-1* specific cDNA using random hexamers and *map1-1* F/R primers. N: reverse transcriptase negative controls. +: *E. ruminantium* gDNA positive control. -: no template PCR control. M: molecular marker.

4.3.6. Transmission experiment 1.

4.3.6.1 Acquisition feeding 1: To generate infected ticks by feeding on experimental animals, sheep were infected with *E. ruminantium* from endothelial cultures and nymphal ticks applied consecutively on days 5, 6 and 7 after inoculation. The clinical responses of test sheep 3148 and 3175 and control sheep 3183 are presented in Annexes 3, 4 and 5 respectively. The 2 test sheep reacted with fever 10 and 11 days post inoculation and were treated on the third consecutive day of fever (days 12 and 13), the control sheep remained healthy throughout the observation period. Using the MAP1-B ELISA, antibodies against *E. ruminantium* were first detected in the test sheep in serum obtained on day 18 post inoculation, while the control sheep 3183 remained sero-negative throughout the 6-week observation period (Annexes 3, 4 and 5). DNA extracted from blood samples collected from the 2 test sheep during the period of fever were tested for *E. ruminantium* by PCR. No bands were detected from sheep 3148 or 3175 using either a single set of MAP1-1 primers or 16S ribosomal genes in a nested PCR (Fig. 4.14). Engorged nymphs were collected after 6 days of feeding on days 11 and 12; this period coincided with the onset of the febrile response in the test sheep (Annexes 3 and 4).

4.3.6.2 Transmission feeding 1: To induce development of *E. ruminantium* infective stages in the vector, the ticks generated in 4.3.6.1 above were placed to feed on naïve animals for 1-5 days. The 2 sheep on which the ticks were fed (sheep 3149

and 3190) and a control sheep (3180) remained clinically normal and sero-negative by MAP1-B ELISA throughout the 6-week observation period. DNA extracted from midguts and salivary glands of female ticks fed on the 3 sheep was amplified by PCR using tick CytC and F3/R1 primers to test for the presence of *E. ruminantium.* A band of 550 bp was amplified from all samples with tick CytC primers indicating successful DNA extraction. No signal was obtained with *E. ruminantium* specific primers F3/R1 in samples from ticks fed on the control sheep (Fig. 4.15), confirming that the ticks were free from *E. ruminantium* infection. These *E. ruminantium*-specific primers gave some faint bands of the expected size in samples from female ticks (from 3149: in all salivary gland samples and in midgut samples from days 2, 3 and 4 after feeding; from 3190: in salivary gland samples from days 1 and 3 after feeding and in salivary glands and midguts from ticks feeding on day 2) but not from male ticks fed on test sheep 3149 and 3190 (Fig. 4.16). Samples of cDNA prepared from midguts and salivary glands from all female tick batches were examined for the presence of the *map1*-cluster transcripts. No bands of the expected size were observed with MAP1-1 primers, although amplification was achieved in all samples when using the 16S ribosomal and Cyt C primers indicating that both tick and *E. ruminantium* RNA was successfully extracted (Fig. 4.17 and Fig. 4.18). The detection of PCR products with 16S ribosomal primers may have been due to the presence of other rickettsial organisms that can infect *A. variegatum* (e.g. *R. africae*; Kelly, 2006). To further investigate whether the ticks were infected with *E. ruminantium*, DNA from tick tissues were analysed using the MAP1-1 nested PCR (Fig. 4.1). Samples of midguts from ticks fed for 2-5 days on sheep 3149, salivary glands from ticks fed for 4-5 days on the same sheep, and salivary glands from ticks

fed for 2 and 5 days on sheep 3190, all gave a clear band of the expected size (182 bp) in the nested PCR (Fig. 4.19). cDNA prepared from the same positive samples was then examined for the presence of *map1-1* transcripts using the MAP1-1 semi-nested PCR (Fig. 4.1). Only samples from midguts of ticks fed for 3, 4 and 5 days and salivary glands from ticks fed for 4 and 5 days on sheep 3149 gave a positive band (Fig. 4.20).

Fig. 4.14

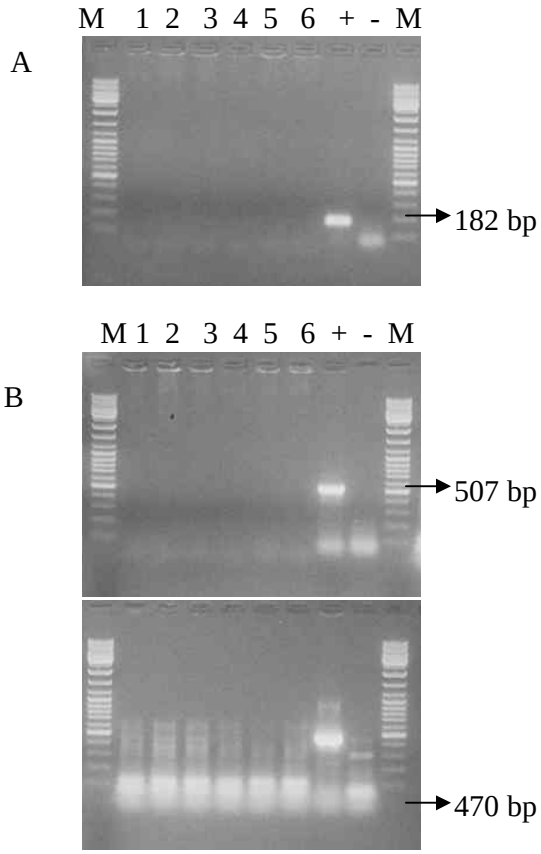


Fig. 4.14 Detection of *E. ruminantium* DNA in blood samples obtained during the first three days of fever of sheep 3148 (lanes 1, 2, 3) and sheep 3175 (lanes 4, 5, 6) using primers F3/R1 (A) and AnEhF1/R1 for the first round (B, top panel) and AnEhF2/R2 for the second round (B, lower panel). +: *E. ruminantium* gDNA positive control. -: no template PCR control. M: molecular marker.

Fig. 4.15

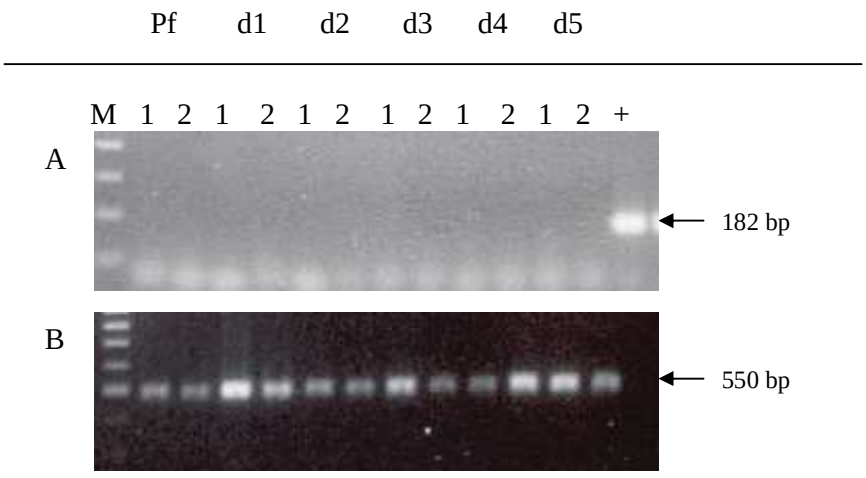


Fig. 4.15: Detection of *E. ruminantium* in ticks fed on control sheep 3180 (trial 1, transmission feeding). DNA extracted from salivary glands (1) and midguts (2) of ticks collected before feeding (Pf) and after 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) days of feeding was amplified with F3/R1 (A) and tick CytC (B) primers. +: *E. ruminantium* gDNA positive control. M: molecular marker.

Fig. 4.16

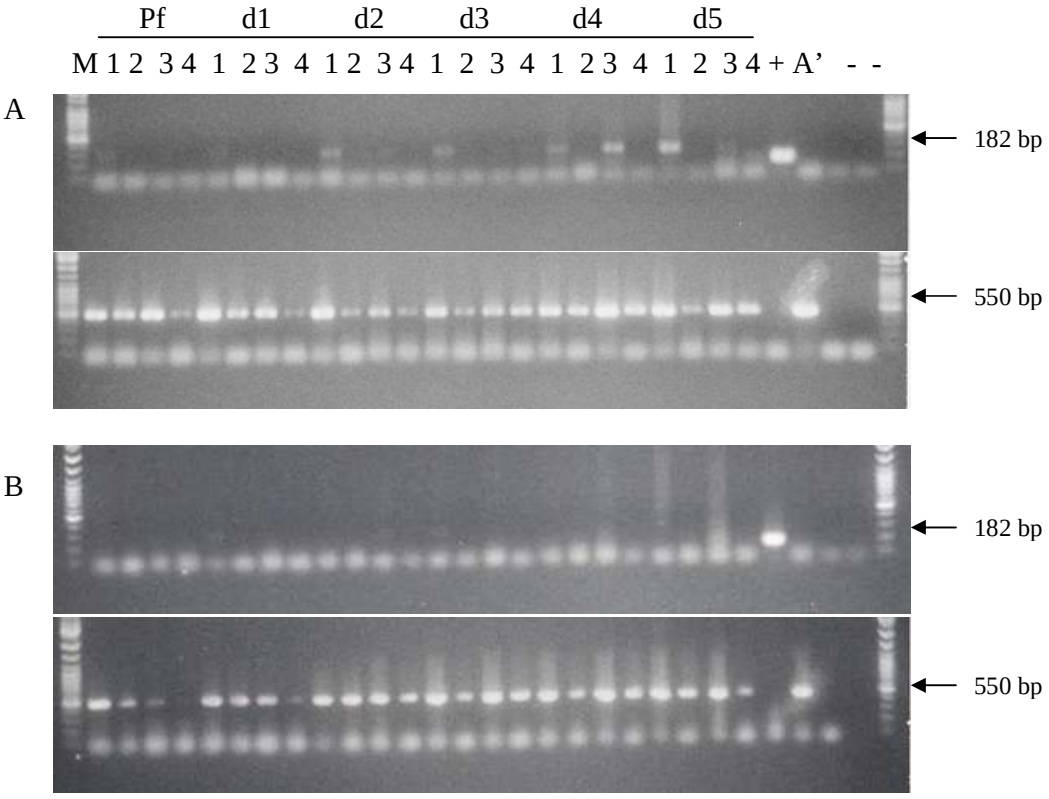


Fig. 4.16: Detection of *E. ruminantium* DNA in salivary glands from female (lane 1) and male (lane 2) ticks and in midguts of female (lane 3) and male (lane 4) ticks collected before feeding (Pf) and on days 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) after attachment on sheep 3149 (A) and 3190 (B) using F3/R1 (A, B top panels) and tick CytC (A, B lower panels) primers. +: *E. ruminantium* gDNA positive control. A': control uninfected AVL/CTVM13 DNA. -: no template PCR control. M: molecular marker.

Fig. 4.17

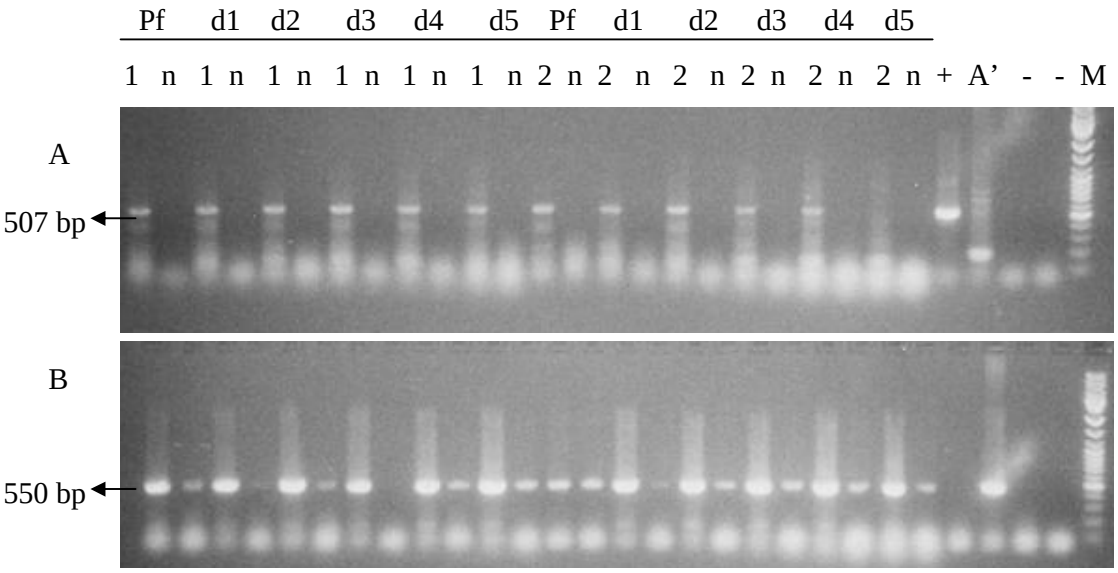


Fig. 4.17 Detection of *E. ruminantium* cDNA in salivary glands (lane 1) and midguts (lane 2) of female ticks collected before feeding (Pf) and after days 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) of feeding on sheep 3149 using primers AnEhF1/R1 (A) and tick CytC (B). n: reverse transcriptase negative control. +: positive *E. ruminantium* gDNA control. A': control uninfected AVL/CTVM13 DNA. -: no template PCR control. M: molecular marker.

4.18

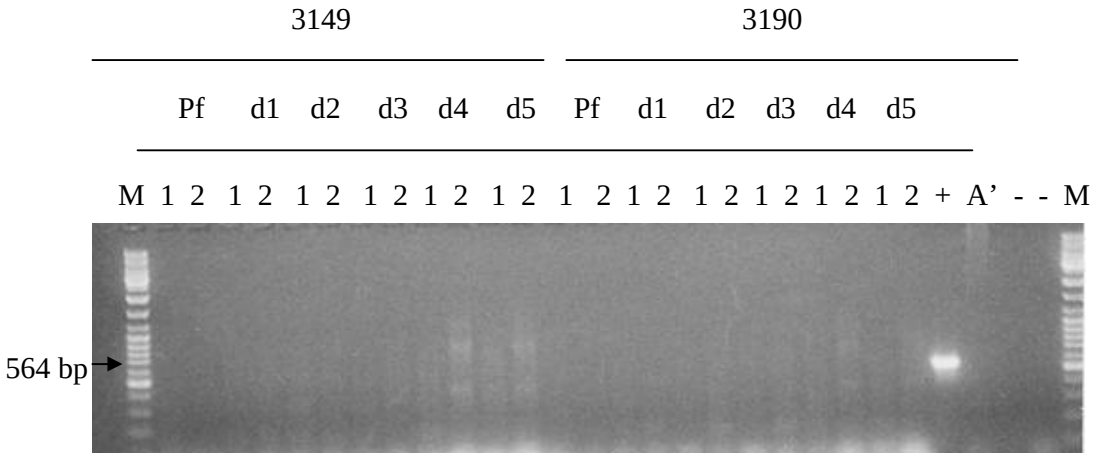


Fig. 4.18: Detection of *map1-1* cDNA in salivary glands (1) and midguts (2) of female ticks collected before feeding (Pf) and after 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) days of feeding on sheep 3149 and 3190 using MAP1-1 F/R specific primers for synthesis of first strand and PCR. +: *E. ruminantium* gDNA positive control. A': control uninfected AVL/CTVM13 DNA. -: no template PCR control. M: molecular marker.

Fig. 4.19

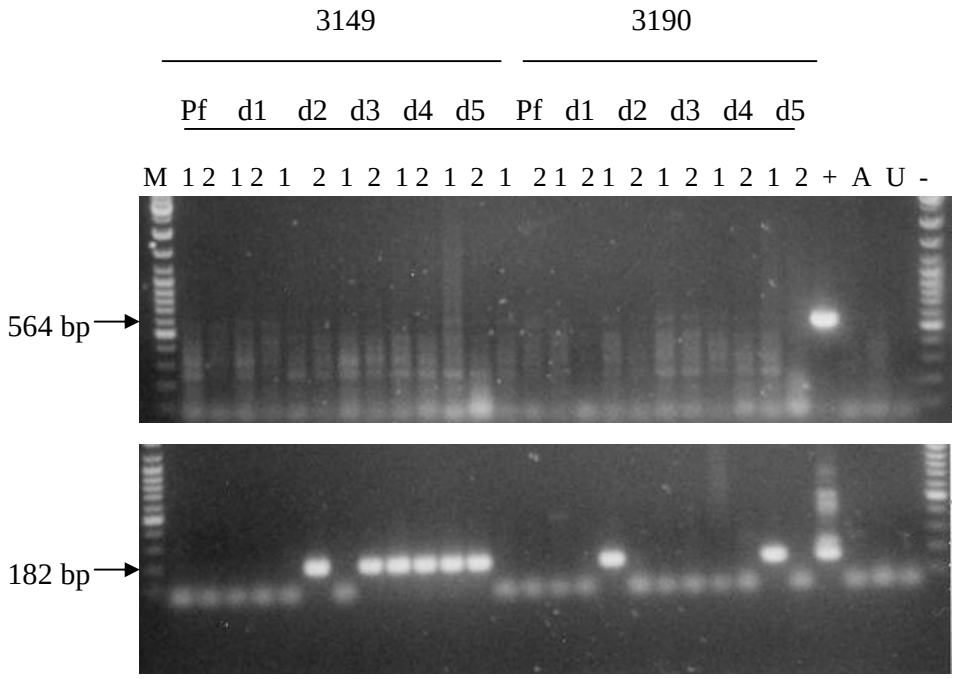


Fig. 4.19: Detection of *map1-1* cDNA in salivary glands (lane 1) and midguts (lane 2) of female ticks collected before feeding (Pf) and after 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) days of feeding on sheep 3149 and 3190 using primers MAP1-1 F/R for the first round (top panel) and F3/R1 for the second round (lower panel). +: *E. ruminantium* gDNA positive control. A': control uninfected AVL/CTVM13 DNA. -: no template PCR control. M: molecular marker.

Fig. 4.20

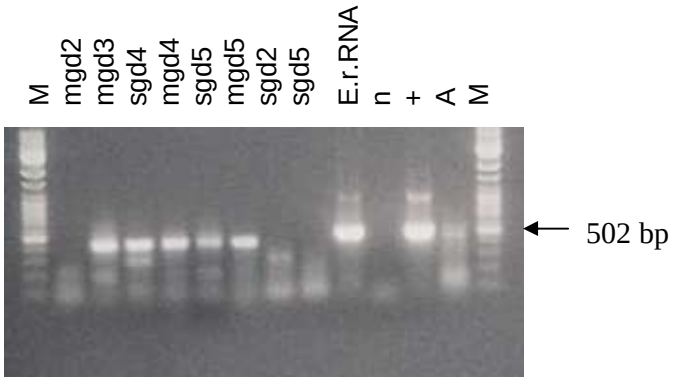


Fig. 4.20: Detection of *E. ruminantium map1-1* transcripts in *E. ruminantium* positive tick samples. The first strands were synthesised with specific primer (MAP1-1R) and amplified by PCR with MAP1-1 F/R (564 bp) to obtain cDNA (data not shown). PCR products were reamplified in a semi-nested PCR using the F3/MAP1-1R primers. Fragments obtained are of the expected size (502 bp). E.r.RNA: DNA-clean RNA from *E. ruminantium*-derived endothelial cells. +: *E. ruminantium* gDNA positive control. A: control uninfected AVL/CTVM13 DNA. -: no template negative PCR control and M: molecular marker.

4.3.7. Transmission experiment 2.

4.3.7.1 Acquisition feeding 2: The lack of a clinical reaction in the sheep in experiment 1 and the difficulty in detecting *E. ruminantium map1-1* transcripts in RNA samples extracted from ticks fed on these sheep indicated a low level of infection in the ticks. Therefore, a second transmission experiment was undertaken, using a larger number of ticks, in an attempt to increase the infection rate in the ticks. The clinical and immune responses of test sheep 3154 were similar to those presented by test sheep in the first experiment (described in section 4.3.6.1) and are presented in Annex 9. Engorged larvae were first collected 7 days after the first batch of ticks was applied to sheep; the tick detachment period from test sheep 3154 coincided with the host febrile response (Annex 9). Samples of DNA extracted from whole blood and buffy coat samples taken from test sheep 3154 on the first three days of fever were tested by PCR for the presence of *E. ruminantium.* Most of the samples gave a faint positive band after one round of amplification by PCR using MAP1-1 F/R primers. Clearer bands were obtained after semi-nested PCR, confirming the presence of *E. ruminantium* in the sheep blood (Fig. 4.21).

4.3.7.2 Transmission feeding 2: To induce development of *E. ruminantium* infective stages in the vector, the ticks generated in 4.3.7.1 above were placed to feed on a naïve sheep. This sheep (3471) remained clinically normal and sero-negative by MAP1-B ELISA throughout the 40-day observation period. Analyses of DNA from whole nymphs fed on this animal by PCR, using *E. ruminantium* F3/R1 and 16S

ribosomal primers, gave no detectable bands in any of the samples; all the samples gave a clear band when amplified with tick primers (Fig. 4.22). Only after semi-nested PCR was carried out, did some of the samples become positive; at least one of the pool samples from pre-fed, d1 and d5 batches gave a clear band (Fig. 4.23). These results indicated that the rate of infection in these ticks was low; therefore no further analysis was done on these samples.

Since test sheep 3471 had not become detectably infected with *E. ruminantium* following application of ticks, it was challenged with stabilate CR366 6 weeks after tick application to determine whether or not it had developed immunity in the absence of any clinical or serological response. Eleven days after challenge, sheep 3471 developed fever and 6 days later showed nervous symptoms typical of heartwater and died. *E. ruminantium* was found in Giemsa-stained brain smears prepared post mortem, and DNA extracted from brain samples was positive by PCR (data not shown).

Fig. 4.21

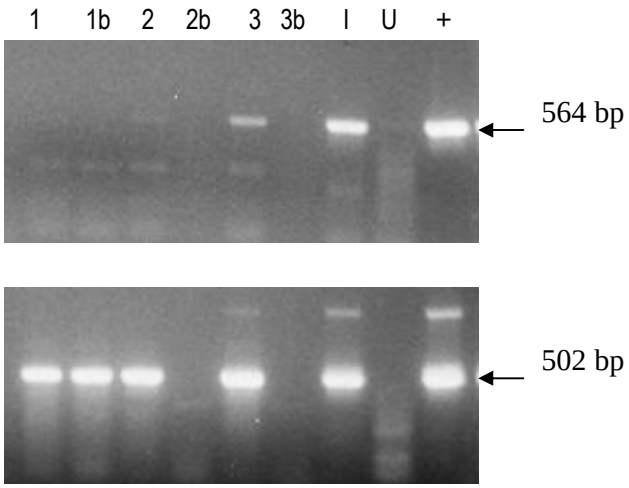


Fig. 4.21: Detection of *E. ruminantium* in blood of sheep 3154 by semi-nested PCR. Lanes contain PCR products of DNA extracted from whole blood samples (1, 2, 3) and buffy coats (1b, 2b, 3b) collected during the first three days of fever of the sheep and amplified with MAP1-1F/R (564 bp) (top panel) and F3/MAP1-1R for the second round (502 bp) (lower panel). I: DNA from *E. ruminantium* infected ticks. U: DNA from uninfected ticks. +: *E. ruminantium* gDNA positive control.

Fig. 4.22

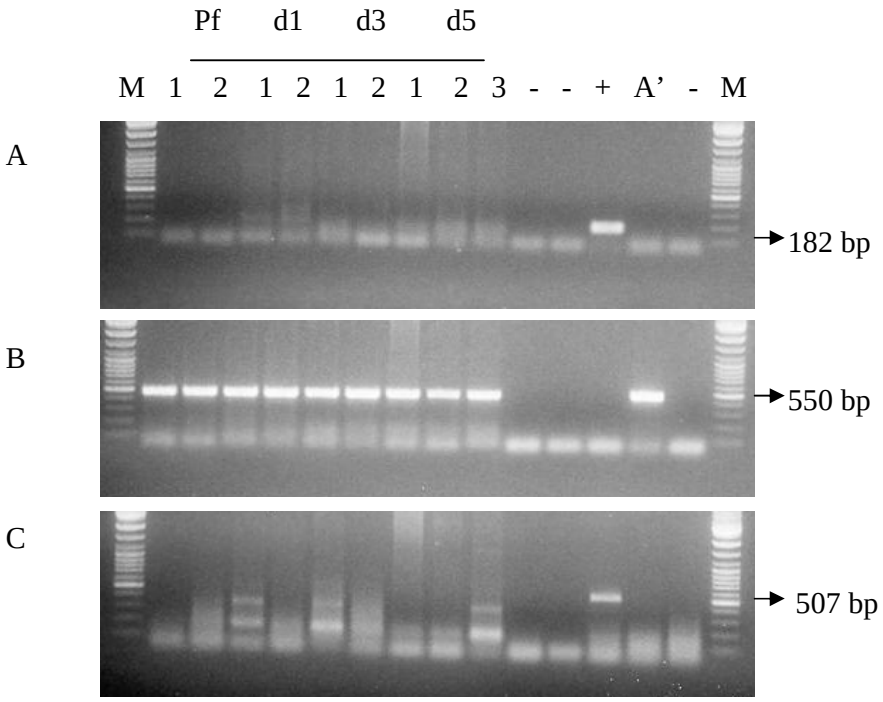


Fig. 4.22: Detection of *E. ruminantium*-specific (A, C) and tick-specific (B) DNA in pools of whole nymphs (lanes 1, 2, 3) collected before feeding (Pf) and after 1 (d1), 3 (d3), and 5 (d5) days of feeding on sheep 3471 using primers F3/R1 (A), tick CytC (B), and AnEhF1/R1 16S ribosomal (C). +: *E. ruminantium* gDNA positive control. A': DNA from uninfected AVL/CTVM13 tick cells. -: no template PCR control. M: molecular marker.

Fig. 4.23

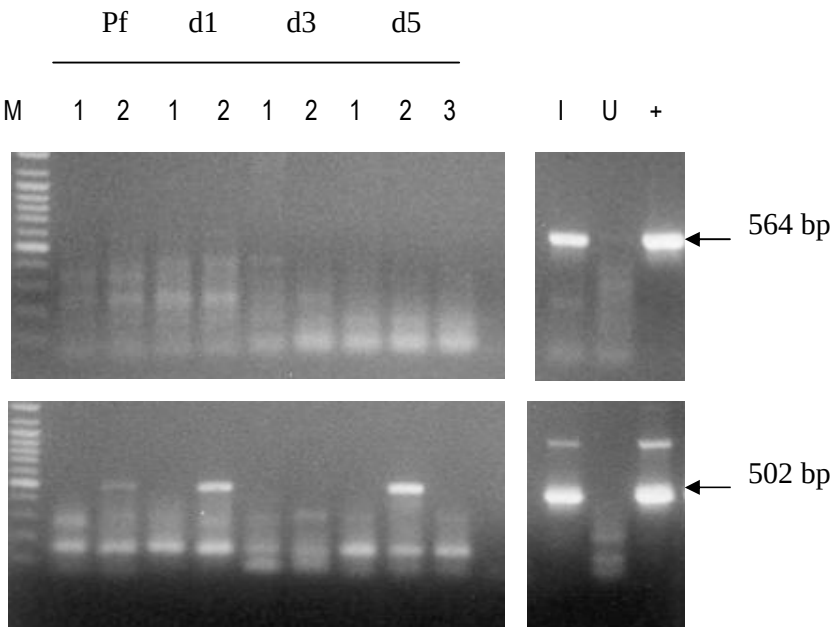


Fig. 4.23: Detection of *E. ruminantium* in ticks fed on sheep 3174 by semi-nested PCR. DNA extracted from pools 1, 2, 3 of whole nymphs collected before feeding (Pf) and after 1 (d1), 3 (d3), and 5 (d5) days of feeding was amplified with MAP1-1F/R (564 bp) (top panel) and F3/MAP1-1R for the second round (502 bp) (lower panel). I: DNA from *E. ruminantium* infected ticks. U: DNA from uninfected ticks. +: *E. ruminantium* gDNA positive control.

4.3.8. Transmission experiment 3.

4.3.8.1 Acquisition feeding 3: Since the cultured subpopulation of the Gardel isolate used in the first 2 transmission experiments was reported to lack one of the genes of the *map1* cluster (*map1-2*, Bekker et al., 2005), a different stabilate of Gardel (CR366) that does not have this gene deletion was used in this third trial. The stabilate CR366 consisted of blood infected with *E. ruminantium* (Gardel) organisms that exhibited virulence similar to the CTVM Gardel population when tested in a preliminary experiment, but that had never been cultured *in vitro*. Sheep 3464 reacted with fever 13 days post inoculation with CR366. Although treatment was started on the third consecutive day of fever (day 15), the sheep died on day 17. Engorged nymphs began detaching from sheep 3464 5 days after the first batch of nymphs was applied and were collected between days 11 and 15 post-inoculation which coincided with the febrile response (Annex 11). DNA was extracted from whole blood and buffy coat samples collected from this sheep during the febrile reaction. PCR products were obtained, using F3/R1 primers, from blood samples taken on the second and third days of fever, indicating that bacteria were present in the sheep blood while the ticks were feeding (Table 2.5). *E. ruminantium* was found in Giemsa-stained brain smears prepared post mortem from sheep 3464, and DNA extracted from brain samples was positive by PCR using MAP1-1F/R primers (data not shown)

4.3.8.2 Transmission feeding 3:

4.3.8.2.1 Experimental infection of ticks: To induce development of *E. ruminantium* infective stages in the vector, the ticks generated in 4.3.8.1 above were placed to feed on a naïve sheep # 3456. This sheep developed fever 13 days after tick application and the next day was found dead. *E. ruminantium* was found in Giemsa-stained brain smears prepared post mortem from sheep 3456, and DNA extracted from brain samples was positive by PCR using MAP1-2F/R primers (data not shown).

DNA extracted from midguts and salivary glands from female ticks, previously fed as nymphs on test sheep 3464, was subjected to PCR using two different combinations of primers (F3/R1 and MAP1-1F/R) specific to the *map1-1* gene. Amplicons of the corresponding expected sizes were observed in all samples of midguts and salivary glands from unfed ticks and ticks fed for 1-5 days on sheep 3456, indicating that all the pools analysed contained *E. ruminantium* (Fig. 4.24).

4.3.8.2.2 Quantification of *E. ruminantium* in tick tissues: DNA samples extracted from midguts and salivary glands of infected ticks, generated in experiment 3 (section 4.3.8) were subjected to analysis by real-time PCR, in order to estimate the number of organisms in different tissues. Five microlitre aliquots of gDNA from pools of dissected tissues from five unfed ticks or five ticks fed for 1-5 days were submitted to quantification using the *map1-1* F3/R1 primers. A 10-fold dilution

series of the plasmid MAP1-1B (containing the *map1-1* gene of *E. ruminantium*) was used to generate a standard reference curve; a linear correlation was found within the dilution range analysed (10^2-10^6 plasmid copies per PCR reaction) in two independent experiments. Based on the knowledge that *map1-1* is a single copy gene, results shown in Figure 4.25 are expressed as the number of bacteria per tick. The results confirmed that *E. ruminantium* was present in midguts and salivary glands of unfed ticks, previously fed as nymphs on sheep 3464, and demonstrated that the number of bacteria increased in both tissues (7-fold in midguts and 10-fold in salivary glands), peaking on day three and decreasing slightly by day five.

4.3.8.2.3 Transcriptional analysis of the *map1* cluster gene members in tick tissues: RT-PCR was performed to study the transcriptional activity of all of the individual genes within the *map1* cluster *in vivo* in ticks and to assess possible differences in transcription of paralogs of the *map1* cluster between midguts and salivary glands in unfed ticks and in ticks fed for different times.

Only 2 out of the 16 paralogs, *map1* and *map1-1*, were found to be transcribed when the whole cluster was analysed (Fig. 4.26). Additional bands, which did not correspond to the expected molecular mass, were seen when using *map1-4, 1-7, 1-9* and *1-10* gene-specific primers. Sequencing of all amplicons demonstrated that only the transcripts from the *map1* and *map1-1* genes were *E. ruminantium*-specific products. DNA template controls are shown for each primer pair to demonstrate the ability of the primers to amplify the target sequence (Fig. 4.26). No transcripts of any of the *map1* paralogs were detected in uninfected tick tissue (Fig. 4.7) and no

amplicons were detected in RT-PCR analysis without reverse transcriptase indicating the absence of gDNA contamination in the RNA preparation (data not shown).

Figure 4.27 shows that transcripts for the *map1-1* gene (a band of the expected size, 550bp) were always found in midguts of unfed and feeding ticks, with the amount of amplified product increasing as the days of feeding progressed. In contrast, *map1* transcripts (a band of the expected size, 685bp) were not found in unfed ticks, but appeared in salivary glands after 2 days of feeding and persisted until day 5. Additional but fainter bands indicate low levels of transcription of the *map1-1* gene in salivary glands during days 2-4 of feeding and the *map1* gene in midguts during days 2-5 of the feeding period (Fig. 4.27).

Fig. 4.24

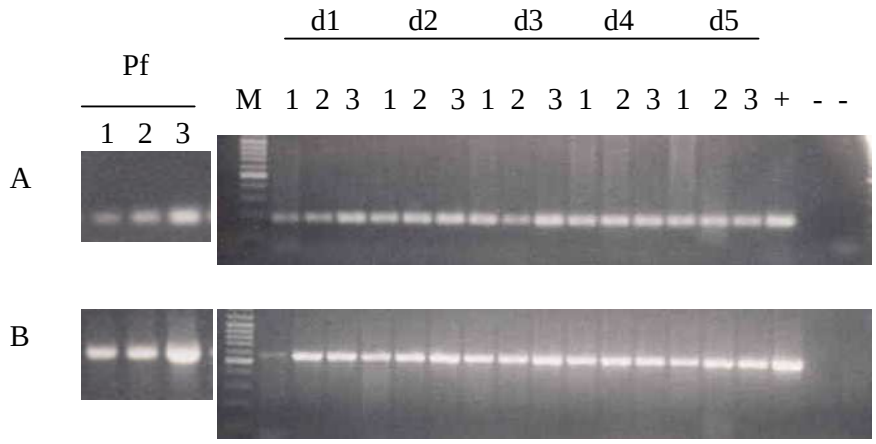


Fig. 4.24: Detection of *E. ruminantium* in ticks fed on sheep 3456. DNA extracted from female tick salivary glands (1) and midguts (2), and from whole male ticks (3) all collected before feeding (Pf) and on days 1 (d1), 2 (d2), 3 (d3), 4 (d4) and 5 (d5) days of feeding, was amplified with F3/R1 (182 bp) (A) and MAP1-1 (564 bp) (B) primers. +: *E. ruminantium* gDNA positive control. -: no template negative PCR control. M: molecular marker.

Fig. 4.25

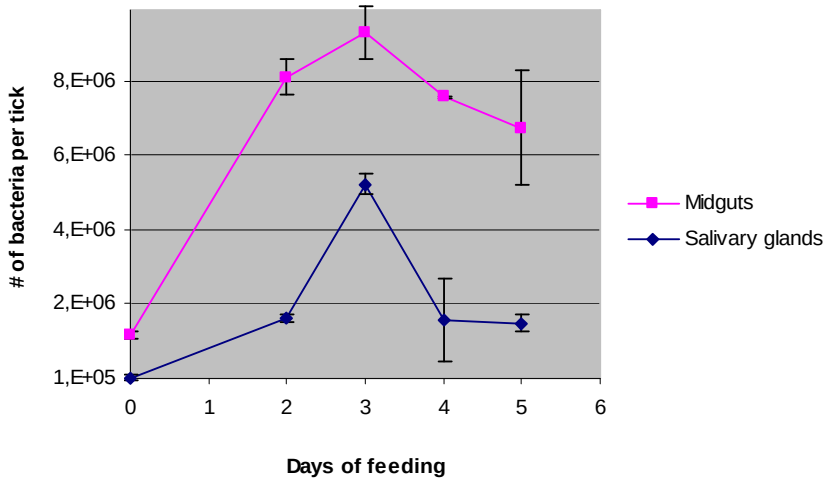


Fig. 4.25: Numbers of *E. ruminantium* in midguts and salivary glands determined by real-time PCR. Bacterial numbers were determined for pools of 5 midguts and 5 salivary gland pairs. Numbers of bacteria are presented as mean per tick plus standard deviation. The mean values were calculated from three readings obtained from two different experiments for each time point.

Fig. 4.26

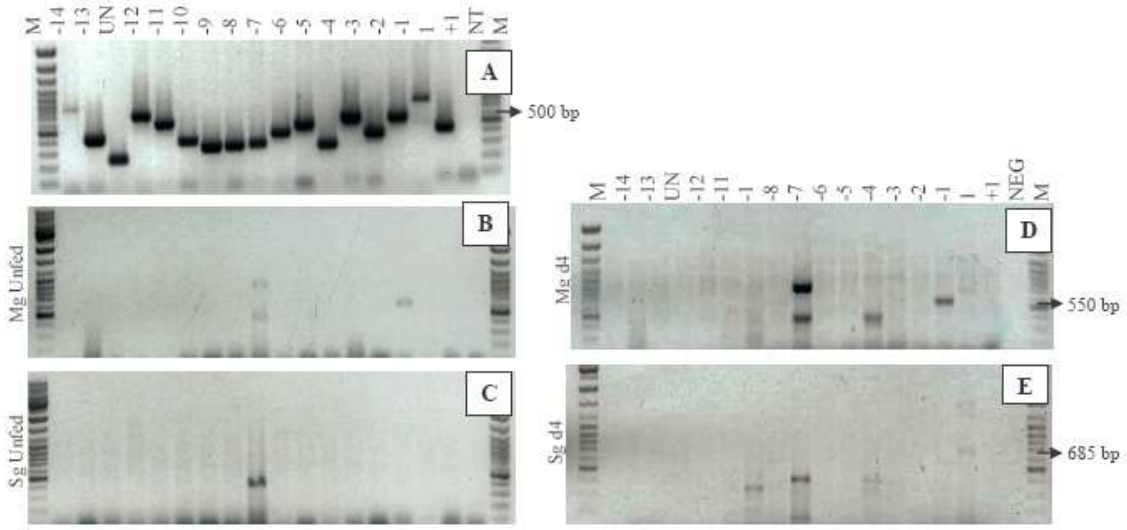


Fig. 4.26: Transcriptional profiles of the *E. ruminantium map1* cluster in unfed and feeding ticks. The panels show (A) PCR products from genomic DNA amplified with *map1* cluster primers for [left to right] *map1-14* to *map1+1*; UN is an unknown gene; (B,C) RT-PCR products from *E. ruminantium*-infected midguts and salivary glands respectively of unfed ticks and (D,E) from midguts and salivary glands respectively of ticks that had fed for 4 days. M: molecular marker, the band that corresponds to 500 bp is indicated in panel A. NT: no template. Nucleotide base pair numbers of specific amplified products corresponding to *map1-1* and *map1* are indicated on the right of panels D and E respectively.

Fig. 4.27

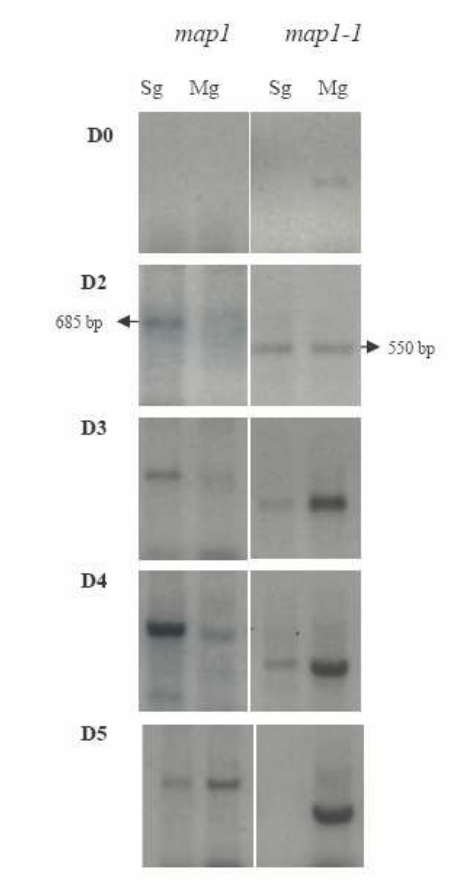


Fig. 4.27: Transcription of *map1* and *map1-1* genes in midguts and salivary glands of unfed and feeding ticks. The panels show RT-PCR products of *E. ruminantium map1* and *map1-1* from midguts (Mg) and salivary glands (Sg) of unfed ticks (D0) or from feeding ticks after 2-5 days of feeding (D2-5). Nucleotide base pair numbers for the amplified genes are indicated with arrows.

4.4 Discussion

The aims of the work described in this chapter were to quantify *E. ruminantium* and to examine the pattern of expression of members of the *map-1* multigene family in midguts and salivary glands of ticks infected with *E. ruminantium* following feeding on infected sheep. Since donor infected sheep were required for these experiments they also provided a further opportunity to investigate PCR methods for detection of infection in sheep blood (Chapter 2).

Transmission experiments involving two steps, first the infection of donor sheep for acquisition of infection by the ticks and second feeding of these ticks, following moulting, on further sheep, were undertaken to examine transmission of infection and development of the *E. ruminantium* organisms in the ticks. Although *E. ruminantium* could be detected in adult ticks after feeding as nymphs on a sheep 3.5 years after infection, confirming the carrier status of that particular sheep (Bekker et al, 2002), under experimental conditions such transmission trials did not consistently provide infected ticks (C. Bekker, personal communication). According to Lounsbury (1900), ticks can acquire infection with *E. ruminantium* while feeding on an animal showing clinical signs of infection. In an experiment involving 2 sheep experimentally infected with heartwater, and on which nymphs were placed at regular intervals following infection, it was found that only those *A. hebraeum* nymphs that engorged during the febrile reaction of the sheep and up to three days after treatment, were able to transmit heartwater in the adult stage. None of the ticks that dropped before the start of the febrile reaction, or those that commenced feeding

during the 20 days thereafter, could transmit the disease in the adult stage (Bezuidenhout, 1987). Moreover, Bezuidenhout (1987), citing unpublished data, reported that the infectivity of goats for ticks was limited to a period extending from 2 days before the temperature reaction to 3 days after its termination. Based on these data, ticks were applied to feed on infected animals during the febrile reaction in the present study.

The time points for sampling of feeding ticks (1-5 days of feeding) were also chosen based on previously published data, which indicated that *E. ruminantium* organisms first develop and replicate in the gut epithelial cells of the tick and subsequently invade and develop in the salivary glands allowing transmission in saliva to the vertebrate host (Prozesky and Du Plessis, 1987). When and how *E. ruminantium* in the tick vector transfers from the midgut to the salivary glands is unknown, although morulae have been seen in haemocytes of adult females after 2 days of feeding (Hart et al., 1991). According to Kocan & Bezuidenhout (1987), *E. ruminantium* colonies were found in midguts of unfed and feeding nymphs (infected as larvae) collected on days 1-4 of feeding but not in those collected on day 5, while colonies in salivary glands were found only in nymphs (infected as larvae) that had fed for 4 days but not in salivary glands of moulting nymphs, unfed nymphs or feeding adults. These results suggested that infection with *E. ruminantium* spreads from midguts to salivary glands only after the tick has matured to the next stage and fed on a new mammalian host. This hypothesis was supported by Bezuidenhout (1987) who reported that feeding on the host is important in stimulating an increase in infectivity as measured by inoculation of homogenates prepared from unfed, feeding or engorged nymphs of *A. hebraeum*, infected as larvae, into susceptible sheep. The

same author (Bezuidenhout, 1987) reported that animals on which infected nymphs had been present for 38 hours or more contracted heartwater, whereas animals on which the nymphs were present for 18 or 26 hours did not become infected. In the case of adult ticks, transmission occurred after the ticks had been present on the animals for 75 hours but not for 20, 26 or 50 hours. The approximate time (6 hours) that ticks take to attach was included in the time periods referred to by these authors. Based on this information, development of infection and expression of *map1* genes during tick feeding were examined over a 5 day feeding period in the present study. Additionally, five days of feeding was considered sufficient for transmission of infection to take place.

The first attempt to obtain infected ticks used *A. variegatum* nymphs fed on sheep infected with the Gardel strain of *E. ruminantium* (CTVM STAB1), which had been previously characterised with respect to kinetics of infection and virulence in sheep (Bell-Sakyi et al., 2002). Based on previous experience from transmission experiments carried out at Utrecht University (C. Bekker, A. Taoufik, F. Jongejan, personal communication), nymphs were applied to the sheep on three consecutive days in order to increase the chance of obtaining ticks that had engorged during the febrile response of the host. Both the infected sheep, 3148 and 3175, developed a clinical reaction and sero-converted, although no signal was detected using an *E. ruminantium*-specific PCR in blood samples collected during the period of febrile reaction of the sheep, around the time of tick detachment. Moreover when, after moulting, the resultant adult ticks were applied to further naïve sheep (3149 and 3190), they failed to transmit infection. Analysis of the extracted DNA from tick tissues by PCR using *map1-1* primers revealed only weak positive reactions in some

samples, although stronger signals were obtained, again only in some samples, using a nested PCR. Despite the apparent low numbers of bacteria in these ticks, an attempt was made to detect at least *map1-1* transcripts in these samples, as *map1-1* transcripts were found to predominate in *E. ruminantium* (Senegal)-infected ticks and *in vitro* cell cultures (Bekker et al., 2002, 2005). A few of the samples gave a positive band indicating the presence of *map1-1* transcripts in tick tissues, but only after semi-nested PCR. The PCR and RT-PCR data, together with the fact that sheep 3149 and 3190 did not become infected after attachment and feeding of acquisition-fed ticks, indicate poor establishment of infection in these ticks, prohibiting detailed studies of *map1* cluster transcription in tick tissues.

Because of the possibility that low levels of infection were in some way related to the batch of ticks used, this experiment was repeated and in addition larger numbers of ticks were used to enhance the possibility of detecting infection in tick tissues. A second experiment used large numbers of larva applied to sheep infected with the CTVM stabilate of Gardel, as in the first experiment. Larvae were used for the acquisition feeding as the smaller size of nymphs compared to adults following feeding made it easier to pool and process large numbers of ticks for extraction of DNA and RNA. Although the sheep inoculated with Gardel stabilate developed clinical disease similar to that observed in the first experiment, the acquisition-fed larval ticks again failed to transmit infection when fed as nymphs on further sheep. Despite the use of larger numbers of ticks to prepare tick tissue pools, only a few of the samples were positive for *E. ruminantium* DNA, and only after nested PCR, indicating very low levels of infection in the ticks.

The failure of ticks in these 2 experiments to transmit infection to naïve sheep, despite the development of a clinical response in the donor sheep similar to that reported previously for the same stabilate (Bell-Sakyi et al., 2002), suggested that this particular population of *E. ruminantium* (Gardel, CTVM STAB1) might have a poor capacity to infect ticks. No tick transmission trials had previously been carried out with the CTVM Gardel isolate (L. Bell-Sakyi, personal communication). Therefore a third trial was carried out, this time using a different stabilate of the Gardel strain of *E. ruminantium*, comprising blood obtained from a sheep infected at Utrecht University (Frans Jongejan, personal communication). Unlike the CTVM Gardel (STAB1), this bacterial population had not previously been cultivated *in vitro.* A preliminary experiment in a susceptible sheep (3471) confirmed that the organisms in this stabilate were virulent and provided information on the kinetics of the clinical response.

In the third experiment, nymphal ticks were fed on a naïve sheep (3464) infected with the Utrecht stabilate of the Gardel strain (CR366), during the period of the febrile response. Following moult, the resultant adult ticks successfully transmitted infection when applied to a second susceptible sheep (3456). This time infection was readily detected in the ticks; all samples of DNA prepared from midguts and salivary glands of the adult ticks fed as nymphs on an infected animal, whether fed or unfed, were found to contain *E. ruminantium* as demonstrated by the presence of a clear PCR product after a single round of amplification with MAP1-1 primers.

This experiment provided materials suitable for a study of the transcriptional activity of the *map1* multigene family in different tissues of *A. variegatum* ticks. Analysis of the samples by RT-PCR revealed that differences in transcription of

map1 genes occur between salivary glands and midguts of both unfed and feeding ticks. Only *map1-1* transcripts were found to be transcribed in midguts of unfed ticks and no transcripts were detected in salivary glands, despite the presence of *E. ruminantium* organisms in the salivary glands before the start of feeding, as determined by real-time PCR. During the tick feeding process, transcripts of the *map1* paralog were always more abundant in the salivary glands of feeding ticks than in their midguts whereas *map1-1* transcripts predominated in the midguts of feeding ticks in comparison to their salivary glands. Although the number of bacteria determined by real-time PCR was always substantially higher in midguts than in salivary glands, higher levels of *map1* RT-PCR products were observed in salivary glands than in midguts. Moreover, the numbers of bacteria in midguts reached peak levels on day 3 and were significantly reduced on day 5, while levels of *map1-1* transcripts in midguts increased over the time course of tick feeding. These observations indicate that the quantities of messenger RNA do not merely reflect differences in the numbers of bacteria but rather show that there are real differences in the levels of transcription. The results are similar to findings reported by IJdo et al. (2002) on *A. phagocytophilum,* which demonstrated differential transcription of members of the P44 multigene family during tick transmission. Several *p44* transcripts were present in salivary glands of transmission feeding nymphs during engorgement but not in unfed nymphs, suggesting that tick engorgement induces the expression of these *p44* genes.

The developmental cycle of *E. ruminantium* in ticks and the route of transmission to the mammalian host are incompletely understood. Light and electron microscopy studies on *Amblyomma* ticks fed on *E. ruminantium*-infected hosts have detected

colonies of *E. ruminantium* in sections of midgut epithelial cells from different developmental stages of unfed and feeding ticks (Cowdry, 1925b; Kocan et al., 1987b; Hart et al., 1991). However, more difficulty has been reported in detecting *E. ruminantium* in salivary glands. *E. ruminantium* colonies were only found in salivary glands of nymphs that had fed for 4 days, but not on days 1-3 or 5 (Kocan & Bezuidenhout, 1987), while no colonies were detected in salivary glands of moulting larvae or nymphs, unfed nymphs or adults that had fed for 1 day or to repletion (Cowdry, 1925b; Kocan et al., 1987a; Hart et al., 1991). Yunker et al (1993), using an *E. ruminantium*-specific DNA probe, detected *E. ruminantium* infection in midguts and salivary glands of unfed adults of *A. hebraeum*. As far as is known, the present study is the first to attempt to quantify the number of *E. ruminantium* in midguts and salivary glands of unfed and feeding ticks. The results from real-time PCR demonstrated firstly, that *E. ruminantium* was already present in both midguts and salivary glands of infected ticks before the start of the transmission feeding and that the number of bacteria increased substantially after 2 days of feeding in both tissues, secondly that the midguts seemed to be the main tissue for colonisation and replication of *E. ruminantium* as the number of bacteria was always higher in midguts than in salivary glands, and thirdly, although the presence of mammal-infective rickettsiae in tick saliva remains to be demonstrated, these results also suggested that transmission of infection in saliva, as opposed to regurgitation of midgut contents, is likely to be the principal route of transmission of *E. ruminantium* to the mammalian host. The observation of a dramatic decrease in the number of bacteria in salivary glands, but not in midguts, by day 4, also supports this last point and is consistent with the finding that *E. ruminantium* require a period of about 75

hours after experimental application to be transmitted by adult ticks (Camus et al., 1996).

A marked increase in the infectivity of *E. ruminantium*-infected ticks during feeding has been reported (Bezuidenhout, 1987). This increase in infectivity may reflect replication leading to a higher number of organisms as reported for *A. phagocytophilum* (Hodzic et al., 1998; Alberdi et al., 1998) and/or unique or enhanced expression of specific pathogen molecules at different stages of feeding. For example, *Borrelia hermsii* expresses serotype 33 of the variable major protein only in ticks (Schwan and Hinnebusch, 1998). When a tick feeds on an infected animal, *B. burgdorferi* within the gut upregulates OspA, which has been shown to be essential for colonisation of ticks (Pal et al., 2004). After moulting, a new bloodmeal triggers replication of the bacterium, downregulation of OspA and upregulation of OspC. OspC has been implicated in facilitating migration of *B. burgdorferi* from the tick midgut to the tick salivary gland, and plays an essential rol during transmission to the mammalian host (Schwan and Piesman, 2002; Ramamoorthi et al., 2005). The detection of differential transcription of *map1* gene family paralogs in different tissues and in unfed ticks compared to feeding ticks suggests that differential expression of outer membrane proteins of *E. ruminantium* in unfed and feeding ticks might be involved in differentiation of organisms to mammalian-infective forms within the tick during feeding.

The difficulty in generating infected ticks following feeding on sheep infected with the CTVM stabilate of the Gardel strain of *E. ruminantium* (STAB1), and the lack of transmission of heartwater to naïve animals by these ticks in experiment 1 and 2 of the present study is similar to observations on goats infected with an attenuated

population of the Gardel isolate (D. Martinez, personal communication). For ethical reasons, sheep 3148, 3175 (used in acquisition feeding 1, trial 1) and 3154 (acquisition feeding 2, trial 2) were treated on the third consecutive day of fever, and thus typical signs of heartwater apart from fever were not observed. Nevertheless, CTVM STAB1 was known from previous studies to be a virulent stabilate. Bell-Sakyi (2004) reported that all sheep challenged with STAB1 showed severe clinical reactions (fever, breathing difficulty, nervous signs) and sheep euthanased on the third day of consecutive day of fever presented hydropericardium and hydrothorax, typical signs of heartwater. A definitive diagnosis of heartwater infection was made in these sheep by finding *E. ruminantium* in brain smears at postmortem. Therefore, the low rate of infection in ticks and the lack of transmissibility of heartwater to naïve animals by ticks reported in this chapter cannot be attributed to the attenuation of organisms in the stabilate used. Moreover, Zweygarth et al. (2004) reported that adult *A. hebraeum* ticks, fed as nymphs on sheep immunised with the attenuated *E. ruminantium* (Welgevonden) isolate, were able to transmit the attenuated stock to a naïve sheep which was subsequently shown to be protected against a lethal homologous needle challenge. Therefore other possibilities must be considered in order to explain the complete failure of ticks to transmit heartwater in transmission trials 1 and 2: The number of *E. ruminantium* organisms present in the blood of animals infected with STAB1 (stabilate used in transmission trials 1 and 2) might have been lower than in animals infected with the blood stabilate CR366. However, real-time PCR analysis of blood from the infected donor sheep in trials 2 (3154, inoculated with CTVM STAB1) and 3 (3464, inoculated with CR366) indicated that the number of bacteria per microlitre of blood in the two sheep by the time the ticks

were feeding was similar (data shown in chapter 2, Table 2.5). A second possibility is that *in vitro* passage of the population of Gardel prior to preparation of the CTVM STAB1 stabilate had resulted in a genetic change that reduced the ability of the organisms to establish infection in the tick vector. Bekker et al. (2005) reported that two subpopulations of the Gardel isolate exhibited a different gene composition in the *map1* cluster, despite the apparent conservation in gene content and organisation of this multigene family among other *E. ruminantium* isolates. The CTVM subpopulation, which was used to prepare CTVM STAB1, showed a recombination between *map1-3* and *map1-2* with the resultant deletion of the entire *map1-2* gene. In contrast, the IBET Gardel subpopulation did not show such a deletion and exhibited the full complement of MAP1 genes (Bekker et al., 2005). The presence of the deletion of the *map1-2* gene in the CTVM STAB1 stabilate used in the present study was confirmed by PCR while stabilate CR366 was shown to have an intact *map1-2* gene (data not shown). Further investigation is needed to determine if this genetic modification or other genetic differences, not yet detected, between these subpopulations of the Gardel isolate are responsible for the difference in tick transmissibility of the organisms.

Chapter 5: **Identification of *E. ruminantium* MAP1 proteins expressed in bovine endothelial and tick cells**

5.1 Introduction

Several recent studies have evaluated the transcriptional activity of multigene families encoding outer membrane proteins in ehrlichial organisms, *in vitro* and *in vivo* (Ohashi et al., 2001; Long et al., 2002 ; Unver et al., 2001; Unver et al., 2002; Felek et al., 2003). With regard to *E. ruminantium*, these reports have revealed the existence of differential transcription of the *map1* multigene family between endothelial and tick cell cultures (van Heerden et al., 2004; Bekker et al., 2005) and *in vivo* between midguts and salivary glands of infected ticks (This study, chapter 4) using RT-PCR methods. Nevertheless, since finding a transcript does not necessarily imply that the mRNA is translated into a protein, it is of significant interest to determine if proteins from the *E. ruminantium map1* cluster, other than MAP1 which is known to be expressed, are actually expressed and if they are differentially expressed in tick and mammalian cell environments.

In the P28 multigene family of *E. chaffeensis* some reports suggest that several proteins are expressed. Zhang et al. (2004a), based on analysis using the peptide enzyme-linked immunosorbant assay, suggested that all 22 paralogs from the *p28-omp1* multigene family were expressed concurrently in persistently *E. chaffeensis*-infected dogs. Singu et al. (2005), using proteomic approaches, reported that only two paralogs, *p28-omp19* and *omp-20* were expressed in *E. chaffeensis* derived from infected macrophage cell cultures, while a different product was found in *E. chaffeensis* derived from tick cells, the protein expressed by the paralog *p28-omp14*.

One of the proteins that has been well characterised in *E. ruminantium* is the major antigenic surface protein MAP1. Jongejan et al. (1991b) raised five monoclonal antibodies against bovine endothelial culture supernatant containing elementary bodies of the Welgevonden isolate. Four of these monoclonal antibodies recognised a 32kDa protein (MAP1) in Western blots prepared from three different stocks of *E. ruminantium.* Electron microscopy and immunogold labelling of *E. ruminantium* organisms *in vitro* using monoclonal antibody 4F10B4 demonstrated that MAP1 is located on the surface of elementary bodies (Jongejan et al., 1991b). The recently published full sequence of the genomes of two different isolates of *E. ruminantium* (Collins et al., 2005; Frutos et al., 2006) suggests that other MAP proteins are likely to be expressed as membrane-bound or secreted proteins since most of them (14 out of 16) contain hydrophobic segments and therefore are predicted to have signal peptides or N-terminal transmembrane domains that could act as signal peptides. The *map1* paralogs are predicted to encode proteins with molecular masses of 24.3 to 35.6 kDa and estimated isoelectric points between 5.7 and 10.0. However, there have been no studies of the expressed proteins.

In the work described in this chapter, a proteomic approach was used to identify host cell-specific *E. ruminantium* proteins encoded by the *map1* cluster, expressed *in vitro* in tick and bovine endothelial cell cultures. In addition, the possible existence of post-translational modifications in the expressed MAP1 proteins was investigated.

5.2 Materials and Methods

5.2.1. Growth and harvest of *E. ruminantium* in bovine endothelial cells and tick cells.

The CTVM Gardel isolate of *E. ruminantium* (Uilenberg et al., 1985) was cultured in bovine endothelial cells (BUE) and tick (IDE8) cells at 37ºC and 31ºC respectively as described previously (Mutunga et al., 1998; Bell-Sakyi et al., 2000a,b). Bacterial growth was monitored by examination of Giemsa-stained cytocentrifuge smears and cultures were harvested as described before in sections 3.2.1 and 3.2.2 of chapter 3. The resultant pellets comprising both infected cells and free *E. ruminantium* organisms were frozen at -20ºC until required for protein extraction. Approximately 10^7 cells, from either uninfected or infected endothelial cell cultures, were used to make each protein stock. For tick cells, approximately 10^6 uninfected or infected cells were harvested to make each tick cell-derived *E. ruminantium* protein stock.

5.2.2. Protein preparation for two-dimensional electrophoresis (2DE).

Soluble and membrane-bound proteins were extracted from *E. ruminantium*-infected and uninfected endothelial and tick cell cultures by resuspending each thawed pellet of lysed cells in 1 ml of 10mM Tris, 10mM NaCl, pH:7.4 (Tris-NaCl) and washing three times in Tris-NaCl followed by centrifugation at 15,000 x g for 20 min at 4ºC. The final pellets were resuspended in lysis buffer comprising Tris-NaCl with 0.5% Nonidet P40 (DBH Laboratories), 2% CHAPS (Sigma) and 1X protease inhibitor (Roche). The suspensions were mixed on a shaker platform at 100 rpm for 45 min at 4ºC and then centrifuged at 15,000 x g for 20 min at 4ºC. The supernatants

were recovered and passed through desalting columns (Pierce) according to the manufacturer's protocol. Desalted proteins were diluted 1:10 in PBS (pH: 7.4) and proteins measured in a Ceres UV 900C ELISA reader (Biotek instruments) according to the Bradford protein assay (Bradford, 1976). In some experiments that required isolation of proteins from solution to remove contaminants (i.e. after enzymatic treatment), protein samples were mixed with TCA (10mM dithiothreitol DTT, 30% Trichloroacetic acid in Millieu water) in a 1:1 ratio, incubated for 45 min at -20ºC and centrifuged for 15,000 x g 15 min at 4ºC. The resultant pellets were washed with acetone (plus 10mM DTT), spun as before and resuspended in rehydration solution (7M urea, 2M thiourea, 4% CHAPS, 0.3% DTT, 0.5% 3-10 NL IPG buffer, 1X proteases inhibitor and trace of bromophenol blue) or Laemmli sample buffer (3X: 2.5 ml 3M Tris pH: 6.8; 2.5 ml glycerol; 3 ml 20% SDS; 1.5 ml β-mercaptoethanol; 0.5 ml Millieu water; trace of bromophenol blue).

5.2.3. SDS-PAGE and immunoblotting. Protein samples (between 5 and 10 μg), were solubilised in 1X Laemmli sample buffer and heated for 5 min at 90ºC. Once cooled, samples were loaded (15-20 μl) in 12.5% acrylamide gels, with a bisacrylamide/acrylamide ratio of 1:37.5 (Bio-Rad Laboratories, Veenendaal, The Netherlands). Electrophoresis was performed in a Hoefer Scientific cell apparatus at 20mAmp per gel for 1 h at room temperature in a 50 mM Tris-glycine buffer. Molecular-mass standards in the range of 14-97 kDa from Amersham Pharmacia were used. Separated proteins were immediately transferred to nitrocellulose (Whatman, Dassel, Germany) or PVDF (Millipore, Amsterdam ZO, The Netherlands) membranes at 38mAmp per gel per hour in a LKB MultiphorII blotter

unit (Pharmacia). To check the efficiency of protein transfer, blots were stained with 8% Direct blue 71 (0.1% DB in water) in 40% ethanol and 10% acetic acid solution. Blots were scanned, destained in destaining solution (96% ethanol, 1M $NaHCO_3$) and kept in deionised water. Non-specific binding was reduced by incubating the membranes for 1 hour at 37ºC in blocking buffer consisting of TBS (20 mM Tris-HCl, pH 9, 0.9% NaCl, 0.05% Tween 20) and 5% skimmed milk (Elk Campina, The Netherlands), added to the TBS buffer just before use. The membranes were incubated with specific antibodies (diluted in 5% skimmed milk in blocking buffer) overnight at 4°C. Pre- and post-infection sera were used at a 1:250 dilution and monoclonal antibodies were used at 1:2,000 unless stated otherwise. Incubation with the appropriate anti-species immunoglobulin secondary antibody conjugated to horseradish peroxidase (DAKO) (diluted 1:2,000 or according to the manufacturer's instructions) was carried out for 1 h at room temperature. The membranes were washed three times with TBS for 5 min after each incubation step. Finally the membranes were incubated with enhanced chemiluminescence detection reagents (Amersham Biosciences) and exposed to X-ray films (Hyperfilm, Amersham Biosciences).

5.2.4. 2DE and Western blot analysis. Between 10 and 30 micrograms of total protein, extracted from *E. ruminantium*-infected and uninfected cells, were mixed with rehydration solution and resolved at 20°C in the first dimension by isoelectric focusing (IEF) in an IPGphor (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) using 7 cm long, precast immobilised nonlinear pH gradient strips, pH 3 to 10 (Amersham Pharmacia Biotech). The IEF parameters were as follows: rehydration of the strips was carried out for 15 hours at 30 volts, followed

by 500 volts for 30 min, 1,000 volts for 30 min and 5,000 volts for 100 min. At the end of the IEF, the strips were equilibrated sequentially for 15 min each in 5 ml of equilibration buffer I (50 mM Tris-HCl [pH 8.8], 6 M urea, 2% sodium dodecyl sulphate [SDS], 30% glycerol and 10 mg/ml DTT) and buffer II (50 mM Tris-HCl [pH 8.8], 6 M urea, 2% SDS, 30% glycerol and 25 mg/ml of iodoacetamide). Subsequently, second-dimension SDS-polyacrylamide gel electrophoresis analysis was performed on the strips in a Hoefer 250 Scientific cell apparatus using 12.5 % polyacrylamide gels (Bio-Rad Laboratories) run at 20mAmp for 1 h at room temperature in a 50 mM Tris-glycine buffer. Molecular-mass standards were used in the range of 10-250 kDa (Bio-Rad). The 2DE resolved gels were stained with either silver nitrate (Merck) or Coomassie blue (Bio-Rad Laboratories) or were used to perform Western blot analysis. Blots containing proteins from *E. ruminantium*-infected or uninfected cell extracts were reacted with sera from sheep inoculated with supernatant from *E. ruminantium*-infected endothelial cells (sheep 3148) or infected tick cell cultures (sheep 4), to identify *E. ruminantium* immunodominant proteins. The monoclonal antibody 4F10B4 (Jongejan et al., 1991b) was used to identify *map1* cluster proteins.

5.2.5. MALDI-TOF MS analysis. Proteins were digested with trypsin (Promega) in 50 mM ammonium bicarbonate (Sigma). Before Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) analysis, peptides were concentrated using µC18-ZipTips (Millipore) and eluted directly on the MALDI-target in 1 µl of a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile. Peptides were analyzed using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems) operated in reflectron mode at 20 kV accelerating

voltage. The resulting peptide mass fingerprint was subjected to an NCBI-nr database search using the Mascot search programs (www.matrixscience.com). Alignments of hit MAP1 family members were performed using the DNASTAR programme and prediction of potential for N- and O-glycosylation sites and phosphorylation sites was performed using the NetNGlyc 1.0, NetOGlyc 3.1 and Net Phos 2.0 servers respectively.

5.2.6. Enzymatic deglycosylation and dephosphorylation. 20 µg of *E. ruminantium*-infected BUE cell proteins in lysis buffer was digested with 5 U of peptide-*N*-glycosidase F, peptide-N^4 (*N*-acetyl-ß-glucosaminyl) asparagine amidase F (PNGase F) (Sigma Chemical) overnight at 37°C. PNGase F hydrolyses the N-linked glycan moieties from asparagine residues in a protein. The dephosphorylation protocol was essentially similar to the deglycosylation protocol except that 15 µg *E. ruminantium*-infected tick cell proteins and λprotein phosphatase (λPPase) (New England Biolabs) were used and the incubation time was reduced to 30 min at 30ºC according to the manufacturer's protocol. λPPase releases phosphate groups from serine, threonine, or tyrosine residues in a protein. After incubation with the enzymes, the proteins were precipitated by TCA as described before (Section 5.2.2) and resuspended in rehydration buffer. The dephosphorylated and deglycosylated samples, along with untreated control samples were resolved and analyzed in silver-stained 2DE gels.

5.2.7. Glycoprotein and phosphoprotein staining. Glycoproteins and phosphoproteins were detected by resolving approximately 30 µg of proteins by 2DE and staining with the Pro-Q Emerald 300 and Pro-Q diamond staining methods

respectively, according to the manufacturer's protocols (Molecular Probes, Eugene, Oregon). Images of the stained gels were captured using an UV transilluminator (UVP Bio imaging System). The gels were restained with silver nitrate to detect total proteins. Candy Cane glycoprotein and Peppermint Stick phosphoprotein molecular weight standards (Molecular Probes, Eugene, Oregon) were used as positive controls for glycoprotein and phosphoprotein detection and protein size determinations.

5.3 Results

5.3.1. Immunodominant *E. ruminantium* proteins. Approximately 10 µg of proteins from uninfected or *E. ruminantium* (Gardel)-infected bovine endothelial (BUE) or tick (IDE8) cells were run on SDS gels and transferred to nitrocellulose membranes. When probed with sera from sheep inoculated with *E. ruminantium*–infected endothelial or tick cells, the post-inoculation sera reacted with antigens only in the lanes containing *E. ruminantium*–infected cell proteins, while the pre-inoculation sera did not reveal any protein bands. Post-inoculation serum from the sheep inoculated with infected endothelial cells reacted with three proteins, of approximately 29 kDa, 30 kDa and 32 kDa, extracted from *E. ruminantium*–infected endothelial cell cultures, but with only one protein (30 kDa) extracted from *E. ruminantium*–infected tick cells. Post-inoculation serum from the sheep inoculated with infected tick cells reacted with two proteins (29 kDa and 30 kDa) in *E. ruminantium*–infected endothelial cells, the smallest (29 kDa) being the most strongly recognized, and with a single protein of approximately 30 kDa in *E. ruminantium*–infected tick cultures (Fig. 5.1).

Fig. 5.1

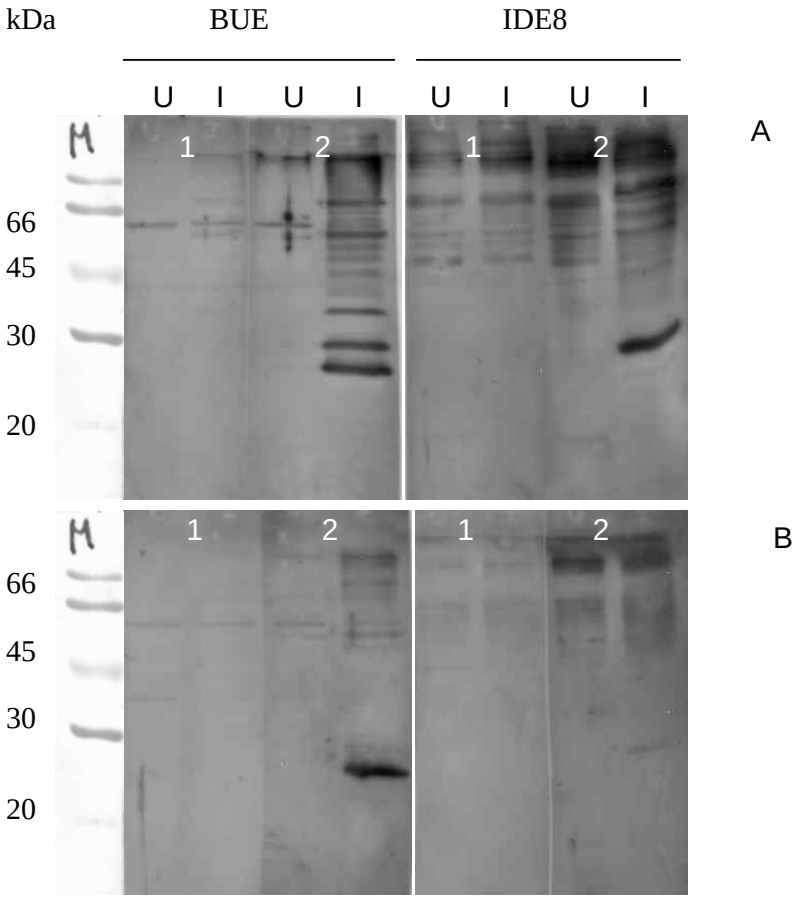


Fig. 5.1: Western blots of 12.5% SDS-PAGE gels containing uninfected (U) and *E. ruminantium*-infected (I) bovine endothelial (BUE) and tick (IDE8) cells. Blots were probed with pre- (1) and post- (2) infection sera of sheep inoculated with *E. ruminantium* in endothelial (A) or tick (B) cells. Molecular size marker (M) in kilodaltons.

5.3.2. Identification of *E. ruminantium* proteins in 2D gels by Western blot analysis. Proteins derived from uninfected and *E. ruminantium*-infected tick and endothelial cell cultures were resolved by two-dimensional gel electrophoresis and silver stained. Purification of the organisms was not considered to be necessary in the present study as differences between the protein spots in gels prepared from uninfected and *E. ruminantium*-infected cells were obviously visible, especially in the area of interest between 25 and 37 kDa (Fig. 5.2). Comparison of the proteomes of *E. ruminantium*–infected endothelial cultures with those derived from tick cells revealed at first sight a higher density of proteins, migrating between pH 4.5 and 5.5, with molecular weights of around 30kDa, in extracts of infected endothelial cells. These proteins were absent in uninfected material while in *E. ruminantium*-derived from tick cells (Fig. 5.2) a single row of proteins of around 30 kDa, migrating widely between pH 4.5 and 6, was observed. Additionally, each spot in the endothelial cell-derived group of proteins showed the same intensity while in the row of proteins from *E. ruminantium* in tick cells, the middle spot showed a higher staining intensity than the others. Further analysis by Western blotting showed that serum from the sheep inoculated with *E. ruminantium*-infected endothelial cells recognised all the spots in this region of the gel. These were the predominant proteins recognised by this serum indicating that they represent immunodominant *E. ruminantium* proteins (Fig. 5.3). In addition, the MAP-1 specific monoclonal antibody 4F10B4 (Jongejan et al., 1991b) was found to react specifically with these particular proteins in gels from infected tick and endothelial cells (Fig. 5.3).

5.3.3. Identification of host cell differences in protein expression of the *E. ruminantium map1* cluster by MALDI-TOF MS analysis.

For mass spectrometry analysis, gels containing 30 μg of proteins were stained with Coomassie blue and the three brightest spots, spots B1, B2, B3 and T1, T2, and T3 (Fig. 5.3) in gels from *E. ruminantium*-infected bovine endothelial cells and tick cells respectively, were excised and subjected to MALDI-TOF MS analysis. The peptide mass lists generated by each spot were subjected to an NCBI-nr database search using the Mascot search programmes. The peptide mass fingerprints generated from spots T1, T2 and T3 all matched with the protein sequence of *E. ruminantium* MAP1-1, while the ones generated by spots B1, B2 and B3 all matched with the protein sequence of the *E. ruminantium* MAP1. These results clearly indicated that the tick cell-derived *E. ruminantium* proteins (spots T1, T2 and T3) were all found to be products of a single gene, *map1-1*, while the proteins extracted from *E. ruminantium*-infected bovine endothelial cell cultures (spots B1, B2 and B3) were the products of a different single gene, *map1*. The analysis was repeated using proteins from infected endothelial and tick cell cultures (Gardel isolate in BUE and IDE8) at different passage levels (at least with a difference of three months in the cultures harvested for the first and second analysis) and the results were the same. An alignment of MAP1 and MAP1-1 proteins and peptides identified by MS, corresponding to all three spots in both *E. ruminantium*-infected endothelial and tick cell samples, are shown in Fig. 5.4. The lack of recognition of peptides in some of the regions not identified in the three MAP1 and MAP1-1 sequences could be due to the occurrence of post-translational modifications because modified peptides are not recognised by MS. Predicted potential *N*- and *O*-linked glycosylation sites (shown

within boxes in Fig. 5.4) and phosphorylation sites (indicated with arrows in Fig. 5.4) were found in these regions, prompting further studies to investigate glycosylation and phosphorylation in these proteins.

Fig. 5.2

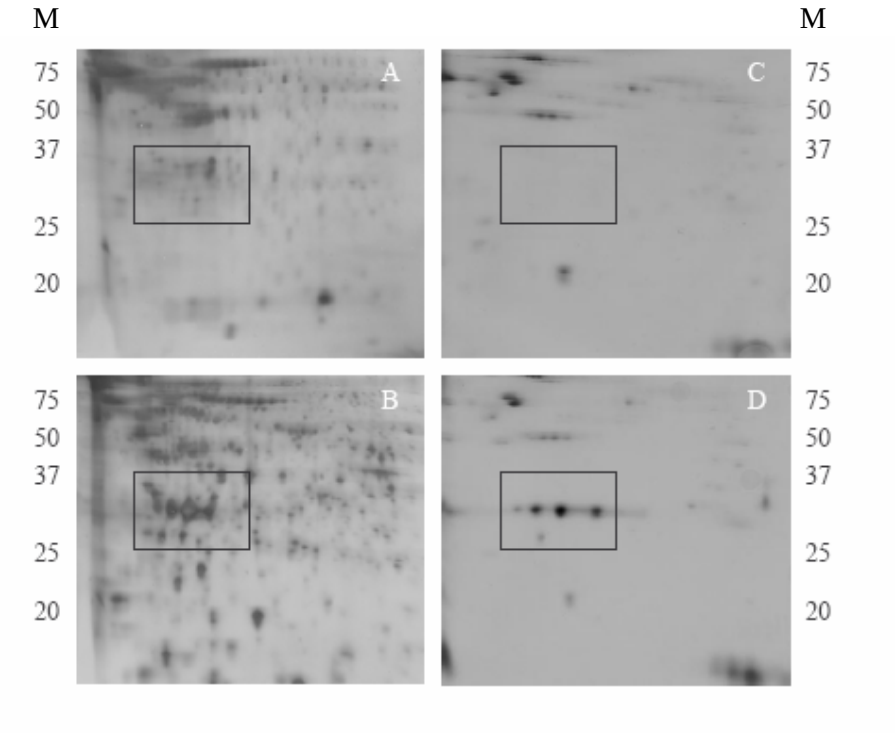


Fig. 5.2: Two-dimensional SDS-PAGE gels of *E. ruminantium* grown *in vitro*. Total protein extracts from uninfected or *E-ruminantium*-infected endothelial (A, B) and tick (C, D) cell cultures were subjected to 2DE analysis and silver-stained. The region of interest (approx. 30 kDa) is surrounded by a box in all panels. M: Molecular masses in kDa.

Fig. 5.3

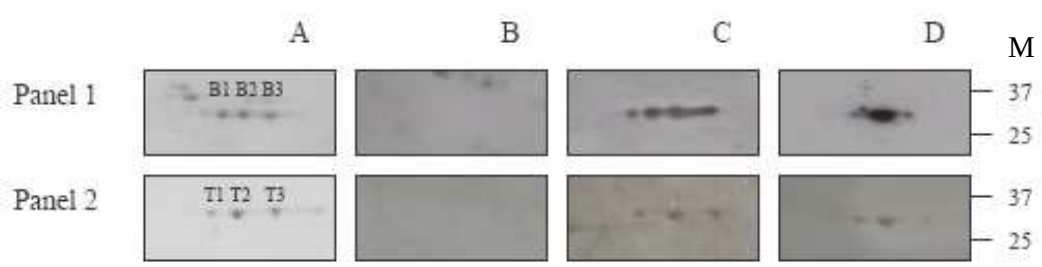


Fig. 5.3: Western blots on *E. ruminantium* 2DE gels. Total protein extracts from *E. ruminantium* grown in endothelial (Panel 1) or tick (Panel 2) cell cultures were resolved by 2DE. Gels (A) were Coomassie blue-stained. Spots analysed by MALDI-TOF MS are indicated as B1, B2, B3 for bovine endothelial cells and T1, T2, T3 for tick cells. Blots from 2DE gels were probed with pre-infection (B) and post-infection (C) sheep serum; and monoclonal antibody 4F10B4 (D). M: Molecular masses in kDa.

Fig. 5.4

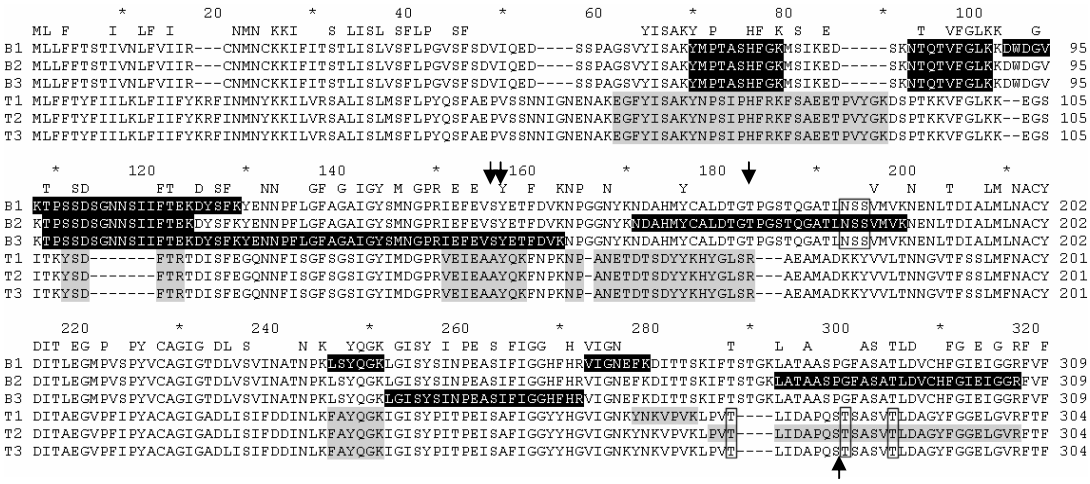


Fig. 5.4: Amino acid sequence alignment of *E. ruminantium* MAP1 (Acc.no. CAI28368) and MAP1-1 (Acc.no. CAI28367) proteins identified by MS analysis. Identical residues between the two proteins are shown in the top row. The identified peptide sequences in B1, B2, B3 (MAP1 proteins) and T1, T2, T3 (MAP1-1 proteins) are shaded black and grey respectively. Predicted *N*- and *O*-linked glycosylation sites are enclosed in boxes. Predicted phosphorylation sites (S, T and Y) are indicated with arrows.

5.3.4. Enzymatic deglycosylation and phosphorylation. An attempt was made to test if the expressed proteins, MAP1 and MAP1-1, were subjected to posttranslational modifications by enzymatic deglycosylation and dephosphorylation. No shifts of spots, either in molecular weight or in pI, were observed in the treated samples in comparison with untreated samples (Fig.5.5). However, as positive controls were not available and therefore not included in these experiments, these results could not be considered conclusive.

5.3.5. Identification of glycoproteins. Further evaluation of the expressed *E. ruminantium* proteins to assess posttranslational modifications was carried out using staining techniques. Staining of periodate-oxidised carbohydrate groups with Pro-Q Emerald 300 stain provided evidence that all three forms of MAP1 expressed in *E. ruminantium*-infected endothelial cells, and all three forms of MAP1-1 expressed in *E. ruminantium*-derived tick cells were glycoproteins (Fig. 5.6). Positive controls for glycosylation, two glycoproteins of 42 kDa and 82 kDa included in the Candy Cane Molecular Marker, were selectively stained while the rest of the proteins of the marker were only visualised after total protein staining. In contrast, staining of the phosphate moiety with the Pro-Q Diamond stain gave negative results in the *E. ruminantium* proteins derived from both endothelial and tick cell cultures. Positive controls, two phosphoproteins of 45 kDa and 23 kDa, gave fluorescent staining while the remaining proteins in the marker lane were observed only after total protein silver staining (Fig. 5.7).

Fig. 5.5

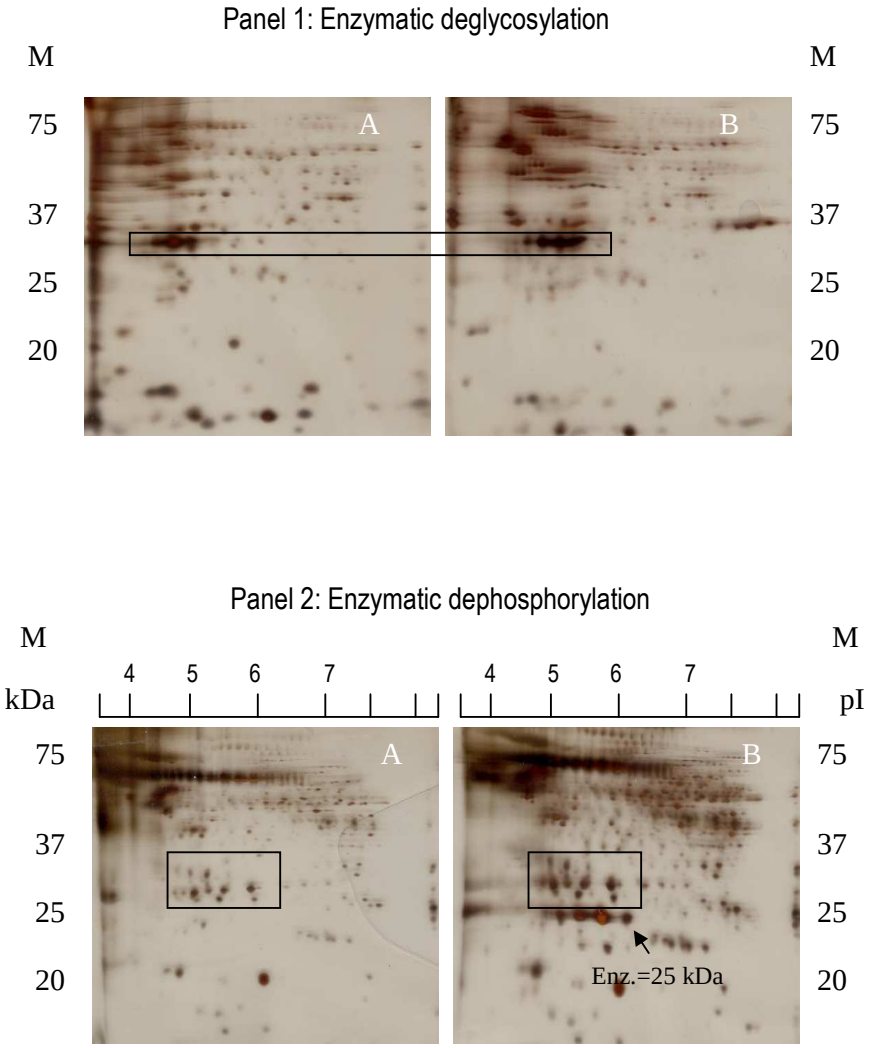


Fig. 5.5: 2DE gels of enzymatic deglycosylation and dephosphorylation of total protein extracts from *E. ruminantium*-infected endothelial (BUE) (Panel 1) and tick (RAN/CTVM3) (Panel 2) before (A) or after treatment (B) with PNGase F (for deglycosylation) and λPPase (for dephosphorylation) respectively. The regions of interest (approx. 30 kDa and 4.5-6.5 pI) are enclosed in rectangles in all panels. M: Molecular masses in kDa. pI: Isoelectric point.

Fig. 5.6

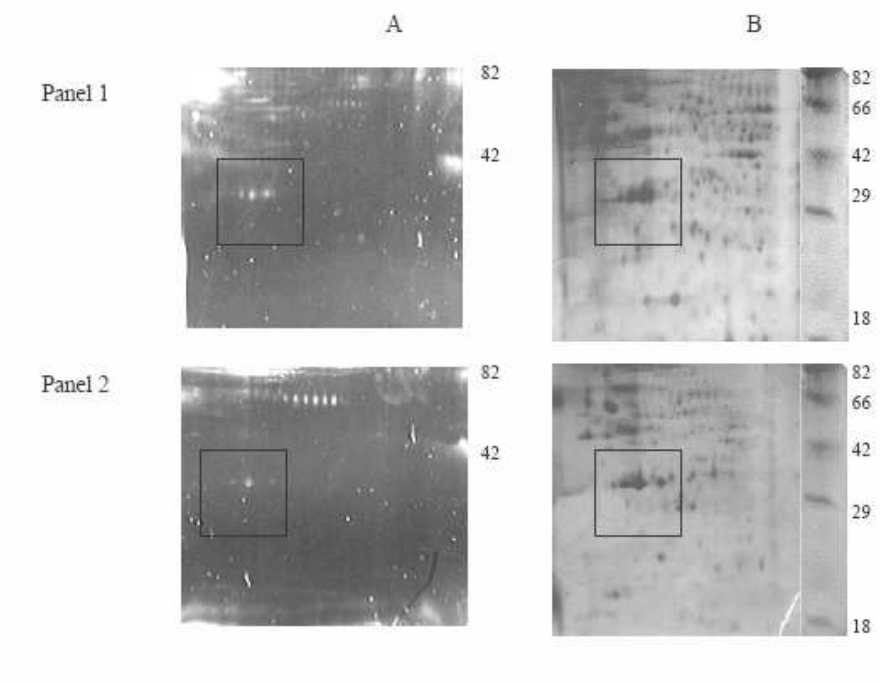


Fig. 5.6: Glycoprotein and total protein staining of *E. ruminantium* 2DE gels. Total protein extracts from *E. ruminantium* grown in endothelial (Panel 1) or tick (Panel 2) cell cultures were resolved by 2DE electrophoresis. Gels were stained for glycoproteins (A) and re-stained with silver nitrate for total protein comparisons (B). Spots of MAP1 (Panel 1) and MAP1-1 (Panel 2) are surrounded by boxes. Glycoprotein molecular weight standards are indicated on the right.

Fig. 5.7

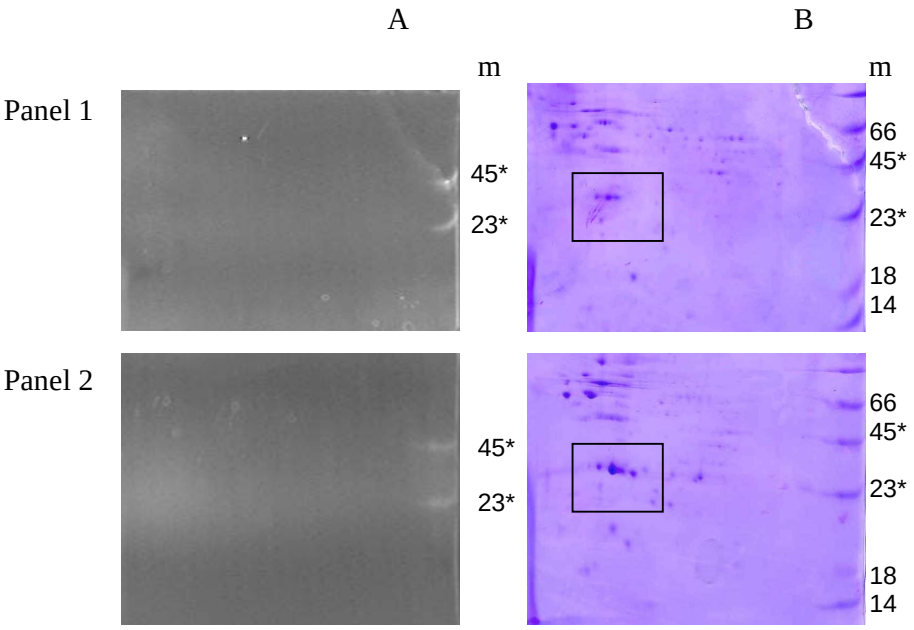


Fig. 5.7: Phosphoproteins (A) and total proteins (B) of *E. ruminantium* grown in endothelial (Panel 1) or tick (Panel 2) cells. Spots of MAP1 (Panel 1) and MAP1-1 (Panel 2) are surrounded by rectangles. m: Glycoprotein molecular weight standards in kDa.

5.4 Discussion

Previous preliminary studies employing 1D SDS-PAGE electrophoresis and Western blotting provided evidence of differential expression of MAP1 proteins in infected endothelial and tick cell cultures (S. Smith, personal communication; Bell-Sakyi, 2004). In Western blots of *E. ruminantium*-infected cell extracts, polyclonal sera revealed that the immunodominant antigen in tick cell stages was not the 30-32 kDa protein found in mammalian stages but a smaller 29 kDa protein. Western blots with the monoclonal antibody 4F10B4, shown previously to react against MAP1 (Jongejan et al., 1991b), identified a 29kDa protein in extracts from *E. ruminantium*-infected tick cell cultures and a 32kDa protein in extracts from *E. ruminantium*-infected endothelial cells. In contrast, the monoclonal antibody 1E5H8 also shown previously to react against MAP1 (Jongejan et al., 1991b) reacted with a 32 kDa *E. ruminantium* protein expressed in endothelial cell cultures but did not recognize any protein in different extracts from *E. ruminantium*-infected tick cell lines IDE8, AVL/CTVM13 and RAN/CTVM3. The differential transcription of *map1* paralogs in *E. ruminantium*-infected endothelial and tick cells *in vitro*, and infected ticks *in vivo*, reported by Bekker et al. (2002, 2005) and further examined and confirmed in the present study (Chapters 3 and 4) also suggested the possibility of differential protein expression in mammalian and tick cells.

In the present study, Western blotting revealed a ladder of proteins, of between 29 and 32 kDa, in *E. ruminantium*-infected endothelial cells, which were recognised by serum from a sheep infected with endothelial cell-derived *E. ruminantium*. Only one

band, of approximately 30 kDa, was recognised by the same serum in *E. ruminantium*-infected tick cells confirming previous results (S. Smith, personal communication) and indicating a difference in protein expression between the two culture systems. A different pattern was observed in blots with serum from a sheep immunised with *E. ruminantium*-infected tick cells (#4). This serum recognised only one protein in the infected tick cell extract, while two proteins were recognised in the infected endothelial cell extracts, suggesting a different pattern of *E. ruminantium* protein expression in tick cells. Since this particular sheep #4 was immune and resistant to challenge is therefore likely that *E. ruminantium* underwent some limited replication in the host despite showing a different antibody profile from that of the sheep inoculated with *E. ruminantium*-infected endothelial cells.

In 2D gels, it was possible that the corresponding protein spots identified in the two cell systems represented different proteins and that recognition by the same sera was due to the presence of different antibody specificities and/or cross-reactivity of the antibodies.

Therefore additional proteomic approaches were used to definitively identify the prominent *E. ruminantium* proteins detected in infected endothelial and tick cell cultures.

Although the profiles of protein spots in 2D gels of organisms grown in mammalian and tick cells were very similar, MALDI-TOF MS analysis revealed that the row of proteins around 30 kDa in extracts from endothelial cells represented expressed products of *map1*, whereas those detected in extracts from tick cells were the products of the *map1-1* gene. The observation that MAP1 and MAP1-1 were both recognised by monoclonal antibody 4F10B4 confirms that the epitope recognised by

this monoclonal antibody is present on both molecules. Our results are similar to those from previous studies on *E. chaffeensis*, where the proteins expressed in infected macrophage cell cultures included the products of the *p28-Omp19* and *p28-Omp20* genes (orthologs of *map1* and *map1+1* respectively), whereas in tick cells the product of a different gene (the *p28-Omp14* ortholog of *map1-1*) was detected (Singu et al., 2005). In the present study, only three of the spots detected in 2D gels were subjected to MS. Thus, the possibility remains that some of the additional spots detected in gels represent different MAP1 proteins. Fourteen out of 16 proteins of the MAP1 family are predicted to have signal peptides and locate to the parasite surface (Collins et al., 2005). Although the extraction protocol should extract both soluble and membrane-bound proteins, it is possible that other MAP1 proteins were not detected in the gels either because of poor solubility or low abundance of expressed protein.

The detection of abundant transcripts for the *map1-1* gene in *E. ruminantium*-infected midguts of *A. variegatum* ticks (Chapter 4) and MAP1-1 proteins in organisms grown in the IDE8 tick cell in the present Chapter, is consistent with the results of previous transcriptional studies in ticks and tick cell lines, which have consistently shown transcription of the *map1-1* gene (Bekker et al., 2002, 2005, Chapter 3), suggesting that this protein may have an important function in growth of the organisms in tick cells. On the other hand, the MAP1 protein was apparently not expressed in organisms grown in the tick embryo-derived cell line IDE8, although transcription of *map1* has been detected in salivary glands of infected feeding ticks (Chapter 4) and in organisms growing in IDE8 tick cell cultures (Chapter 3). Real-time RT-PCR will be required to examine the levels of MAP1 transcripts in IDE8.

Absence or low abundance of MAP1 proteins in IDE8 possibly indicates that protein expression of MAP1 may be dependent on growth within a differentiated cell type in the salivary glands.

An interesting observation was the identification of the MAP1 or MAP1-1 proteins in spots with the same molecular size but differing p*I* in 2D gels. The most likely explanation for this finding is the presence of forms of the proteins with different post-translational modifications. For instance, different degrees of glycosylation at a single site in a single protein can result in a serie of protein spots that separate on the basis of different isoelectric point and/or molecular mass in 2D gels (Sickmann et al., 2002). In addition, analysis of the predicted amino acid sequences of MAP1 and MAP1-1 proteins revealed potential glycosylation sites which tended to be in regions for which no peptides were identified by mass spectrometry.

An investigation was carried out to determine whether or not the expressed MAP1 family proteins were glycosylated and/or phosphorylated. Use of a carbohydrate-specific staining method provided evidence that the three forms of MAP1 expressed in *E. ruminantium* derived from endothelial cells, and the three forms of MAP1-1 expressed in *E. ruminantium* derived from tick cells, were glycoproteins. However, when an enzymatic deglycosylation was attempted, no shift in molecular weight was observed in any of the spots in 2D gels after silver staining. As PNGase F hydrolyses the N-linked glycan moieties from asparagine in a protein (Singu et al., 2005), the absence of a detectable effect of this enzyme on the MAP1 proteins may reflect the use of O-linked glycosylation sites. Another explanation for the lack of detectable differences in size of the proteins after enzymatic treatment may be due to the difficulty in observing in gels small differences in molecular size as MAP1 and

MAP1-1 proteins do not exhibit larger-than-predicted molecular masses in SDS-PAGE (van Heerden et al., 2004). Garcia-Garcia et al. (2004) reported that *A. marginale* MSP1a and MSP1b are glycosylated. However, although MSP1a and MSP1b were predicted to have O- and N-glycosylation sites respectively, enzymatic deglycosylation analysis by treatment with N-glycosidase F and O-glycosidase DS, enzymes specific for carbohydrate moieties commonly present in N- and O-glycoproteins, did not reveal a change in protein migration in SDS-PAGE gels. The authors considered that unusual modifications (such as absence of N-acetylgalactosamine or N-acetylglucosamine), known to occur in other bacterial glycoproteins, might be present in these proteins. Furthermore, McBride et al. (2000b, 2003) reported glycosylation of immunodominant proteins in *Ehrlichia* spp. (P120 and P156 in *E. chaffeensis* and P140 and P153 in *E. canis*) and suggested that glycosylation of P120 and P140 may be O-linked, based on the presence of only one predicted site for N-linked glycosylation plus the lack of effect of N-glycosidase F. In contrast, Singu et al. (2005) reported that glycosylation in *E. chaffeensis* P28-OMP1 appeared to be N-linked since, after enzymatic deglycosylation of these proteins using N-glycosidase F and analysis by 2DE and Western blots, the molecular masses of the OMP1 proteins were shown to decrease by approximately 2 kDa. MAP1 and MAP1-1 are each predicted to have two sites for N-linked glycosylation and multiple putative sites for O-linked glycosylation, supporting the hypothesis of presence of O-linked-glycans in these proteins. Despite several reports of glycoproteins in ehrlichial pathogens, glycosyltransferases have not yet been identified in the annotation of ehrliclial genomes.

Singu et al. (2005) reported *E. chaffeensis* OMP1 proteins to be phosphorylated using staining and enzymatic digestion methods for identification of proteins containing phosphorylated serine, threonine and tyrosine residues. MAP1 and MAP1-1 each have several predicted phosphorylation sites in these residues. However, neither staining methods nor enzymatic treatment indicated that the proteins are phosphorylated; nevertheless, further studies are required before drawing a firm conclusion.

Chapter 6: Attempts to identify differentially expressed genes of *E. ruminantium* grown in endothelial and tick cells by suppression subtractive hybridisation (SSH)

6.1 Introduction

Alterations in bacterial gene expression are associated with biological and pathological processes. The identification of differentially expressed genes often leads to greater insight into the molecular mechanisms for bacterial pathogenesis.

Differential transcription of the *map1* multigene family has been demonstrated *in vitro* in tick and mammalian cell cultures (van Heerden et al., 2004; Bekker et al., 2005) and *in vivo* between midguts and salivary glands of infected ticks (this study). These data suggest that transcriptional changes in the *E. ruminantium map1* cluster may be necessary for a particular phase of infection.

Since sequencing of the genome of the Gardel isolate of *E. ruminantium* has been completed (Frutos et al., 2004), studies to detect differential transcription of many more genes have become possible, and may provide valuable information regarding the molecular basis of pathogenicity of *E. ruminantium*.

Suppression subtractive hybridisation (SSH) is a technique designed to identify differences in gene expression between two cell populations by isolating the mRNAs or derived cDNA uniquely present in one cell type. The cDNA in which specific transcripts are to be found is called tester, and the reference cDNA is called driver. Essentially, cDNA from a driver preparation is hybridised in excess against a second population (the tester), to remove common (hybridising) sequences, thereby enriching for target sequences unique to the tester population. The process can be divided into three main phases: The generation of PCR amplicons representative of the RNA isolated from given bacterial populations; the hybridisation of tester and

driver populations and enrichment of unique tester sequences by PCR targeted to linker-ligated tester molecules; and the cloning and screening of the resultant products (Winstanley 2002; Diatchenko 1996). A schematic representation of SSH is shown in Fig. 6.1.

In an attempt to sample a large portion of the *E. ruminantium* genome for genes differentially expressed between *E. ruminantium* (Gardel) grown in endothelial and tick cells, a SSH method was applied.

6.2 Materials and Methods

6.2.1. Growth and harvest of *E. ruminantium* in bovine endothelial and tick cells.

Uninfected and *E. ruminantium*-infected bovine pulmonary artery (BPC) cells and tick cell (IDE8 and AVL/CTVM13) cultures were harvested as described previously in sections 3.2.1 and 3.2.2 of chapter 3. The *E. ruminantium*-infected cell pellets were immediately submitted to a bacterial purification protocol, while pellets of uninfected endothelial and tick cells were used directly for extraction of total host RNA.

6.2.2. Purification of *E. ruminantium* from tick and endothelial cell cultures.

Pellets were incubated with 500 μg/ml trypsin solution (Sigma) and digestion was carried out for 20 min at 37°C. Mechanical disruption of host cells was achieved by repeated passage through a 26-gauge needle. Low speed centrifugation was used to remove most of the host cell debris. The resultant bacterial suspension was treated with RNAse (Sigma) to reduce host RNA contamination prior to extraction of total *E. ruminantium* RNA.

6.2.3. RNA isolation.

Total RNA from *E. ruminantium* was extracted from purified bacterial pellets using the tissue protocol provided with the QIAamp extraction kit (Qiagen). Total RNA preparations from uninfected cells and *E. ruminantium* were treated after elution from the column with RNase-free DNase I (Sigma) following the manufacturer's instructions. RNA suspensions were quantified in a spectrophotometer at 260λ, while purity was confirmed by a spectrophotometric

A260/A280 ratio of >1.8. To assess the quality of the RNA, a small aliquot was analysed by non-denaturing agarose gel (1.5%) electrophoresis. RNA preparations were stored at -70°C.

6.2.4. Reverse transcriptase PCR (RT-PCR). In order to confirm that the RNA preparations were free from DNA contamination, synthesis of cDNA was performed from *E. ruminantium* and host RNA samples using a first strand cDNA synthesis system (Superscript, Invitrogen) and random hexamers. To generate a specific second strand of cDNA and to check for the presence of bacterial and host cell transcripts, 0.4 μM of primer pairs specific for *E. ruminantium map1* (g-for/g-rev), *E. ruminantium map1-1* (F3/R1), *A. variegatum* cyt C gene (CytC F/R) and bovine beta-actin (Bov F/R; kindly provided by Dr. Tracey Coffey from the Biotechnology and Biological Sciences Research Council) were used. Primer sequences and fragment sizes are described in Table 2.3.

6.2.5. Separation of bacterial mRNA. As ribosomal RNA constitutes approximately 85% of the total RNA (Bowler et al., 1999), an attempt to separate mRNA from total bacterial RNA was made using the MICROBExpress kit from Ambion, according to the manufacturer's instructions. This kit is based on removal of 16S and 23S rRNA sequences by hybridisation with 16S/23S oligonucleotides attached to beads. To check the efficiency of the kit with *E. ruminantium*, primer pairs specific to ehrlichial 16S and 23S ribosomal RNA were designed (Table 2.3). These primers were also used to generate PCR products for removal of 16S and 23S rRNA sequences.

6.2.6. Preparation of tester and driver for the first and second stage of SSH.

cDNA from *E. ruminantium* growing in endothelial (BPC) and tick (AVL/CTVM13) cells was synthesised using the Universal Riboclone cDNA Synthesis System (Promega) and random primers, following the manufacturer's instructions, from approximately 1µg of DNAfree-total RNA. The resulting cDNA was digested with DpnII (New England Biolabs) and ligated to 10 pmol oligonucleotides P12 (GATCCGTTCATG) and P24 (ACCGACGTCGACTATCCATGAACG) using T4 Ligase (New England Biolabs). The ligation mixture was purified using the QIAquick PCR Purification kit (Qiagen) to remove excess oligonucleotides. The linker-ligated cDNA was initially incubated at 68°C for 5 min using Taq DNA polymerase (Invitrogen) and then amplified by PCR for 25 cycles with the same Taq and the P24 linker-specific primer. The addition of linkers to cDNA followed by PCR allowed amplification of sequences before the subtraction procedure.

6.2.7. Suppression subtractive hybridisation (SSH).

Two stages of SSH were carried out in this study. A schematic diagram shows the combination of drivers and testers used in the first and second stage of subtraction (Fig. 6.2). In the first stage, *E. ruminantium* linker-ligated cDNA grown in either tick or endothelial cells was used as tester, versus total RNA from uninfected tick or endothelial cells respectively, as driver, in an attempt to get rid of contaminating host cell sequences from the cDNA prepared from extracted *E. ruminantium* (Fig. 6.2). In the second stage, the products obtained in the first stage of SSH from organisms

isolated from tick cells ("tickproduct1 and 2") and endothelial cells ("BPCproduct1 and 2") (Fig. 6.2), were used either as tester or driver to identify genes differentially expressed in tick cells, when "tickproduct1 and 2" were linker-ligated and used as tester (SSH "A" and "C"), and to identify genes differentially expressed in endothelial cells, when "BPCproduct1" was linker-ligated and used as tester (SSH "B"). Only linker-ligated tester sequences would be enriched in the PCR after subtractive hybridisation (Fig. 6.1).

The first stage of SSH was performed as follows: 4 μg of tester were mixed with 40μg of driver, extracted with Tris/HCl-saturated phenol/chloroform/isoamyl alcohol (25:24:1), pH 8, precipitated with ethanol and resuspended in hybridisation buffer (15 mM EPPS, 1.5 mM EDTA, pH 8). The hybridisation mix was incubated at 42°C for 20h and then subjected to digestion with 70U of mung bean nuclease (MBN) (Promega) for 30 min at 30°C to remove all single stranded DNA. Then the mixture was amplified in a 100 μl PCR reaction with P24 primer and 5 units of Taq polymerase. Two rounds of subtraction were completed during the first stage (Fig. 6.2). PCR products resulting from the first stage of subtraction were used as tester and driver for the second stage and subtraction was performed as above. Three rounds of subtraction were completed for "A" and "B" in a tester:driver ratio of 1:25. Two rounds of subtraction were completed for "C" with 1:25 and 1:5 tester:driver ratios respectively.

6.2.8. Construction and clonal analysis of a subtractive library.

SSH products from "A", "B" and "C" were purified using the QIAquick PCR Purification kit (Qiagen), inserted into the pGEM®-T Easy Vector System II

(Promega) and transformed into competent *Escherichia coli* JM109 cells. Screening for inserts, between 200-600bp, was carried out by PCR, in 105, 133 and 110 white positive colonies from SSH products “A”, “B” and “C” respectively, using primers specific to the pGEM-easy vector (T7 and SP6) according to the manufacturer’s instructions. Colonies containing inserts larger than 250 bp were selected, grown overnight at 37°C, and stored at -70°C in glycerol. From these glycerol stocks, twenty-two colonies from SSH products “A”, “B” and “C” were picked at random, grown overnight at 37°C, and submitted to isolation of the vector (Promega Wizard® Plus Minipreps DNA Purification System) for further sequencing of the inserts. Sequencing was carried out at the Functional Genomics Unit of the Moredun Research Institute and sequences were analysed using the programme Seqman-DNA star.

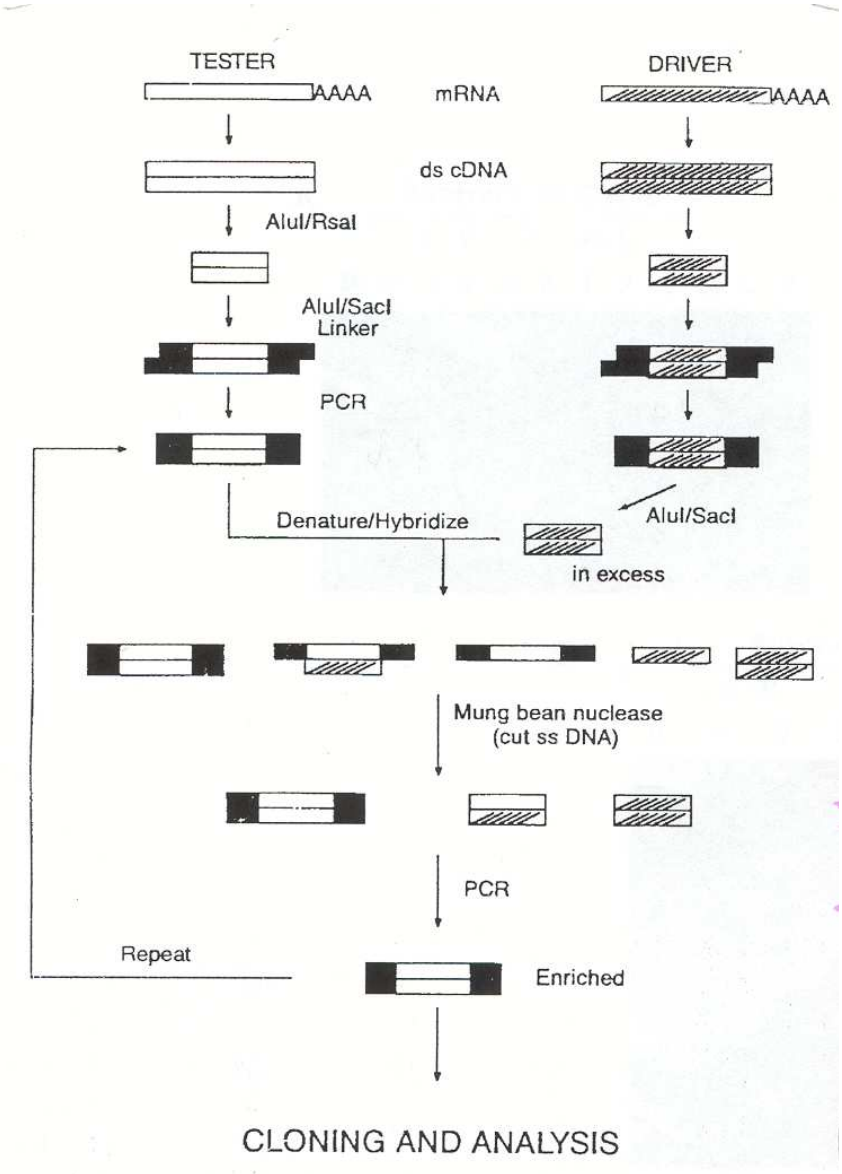


Fig. 6.1: Schematic illustration of suppression subtractive hybridisation taken from Yang and Sytowski, 1996.

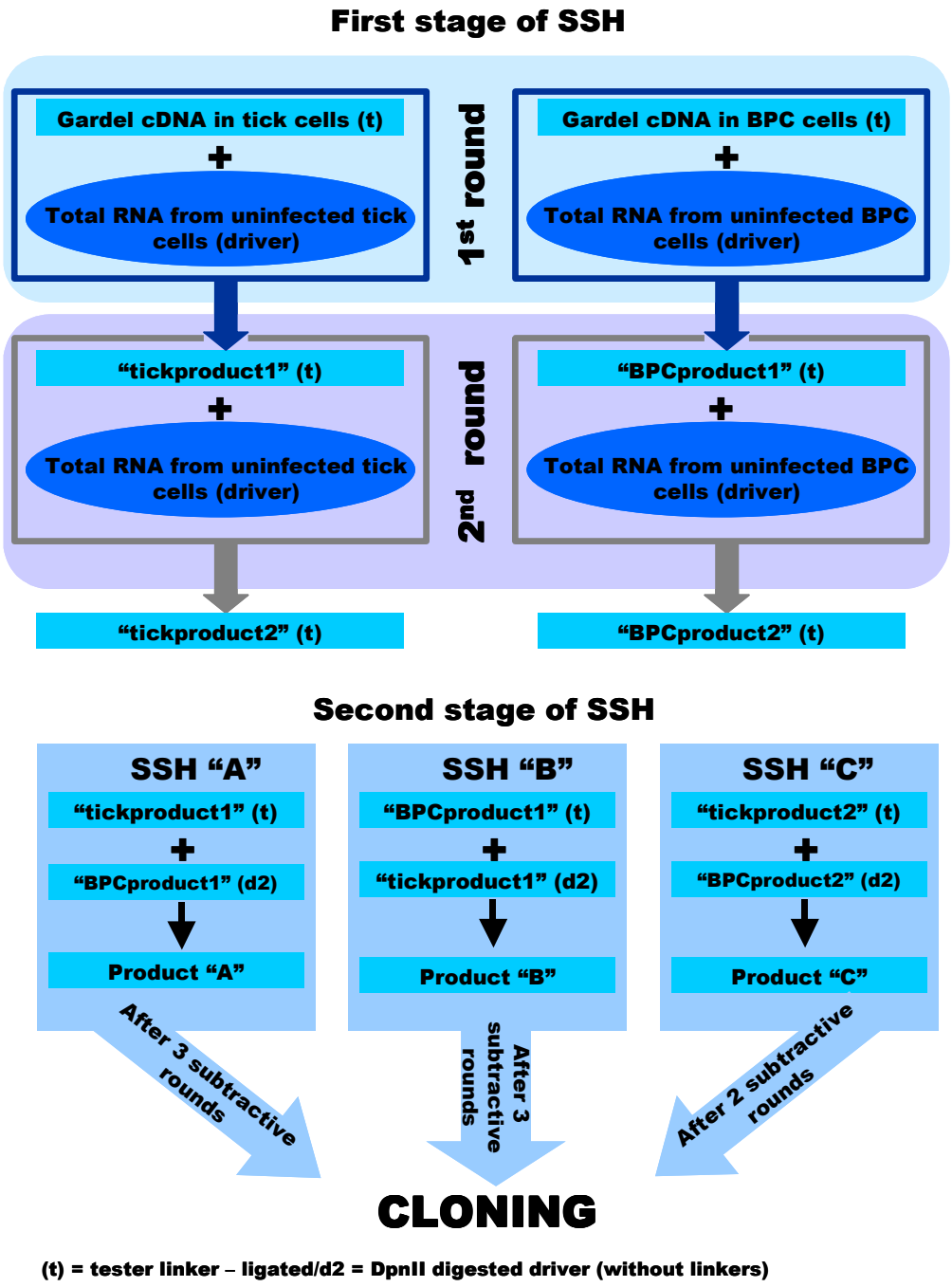


Fig. 6.2: Schematic diagram of applied subtractive steps (Details in section 6.2.7)

6.3 Results

6.3.1. Purification of *E. ruminantium* from tick and endothelial cell cultures.

Low speed centrifugation was used to clear out most of the host cell debris, yielding ehrlichial organisms that were relatively free of host cell material, as judged by microscopic observation of purified preparations in Giemsa-stained cytocentrifuge smears (Figs. 6.3 and 6.4 A,B).

6.3.2. *E. ruminantium* RNA isolation.

Prior to extraction of *E. ruminantium* total RNA, the resultant bacterial suspension was treated with RNAse to reduce contamination with host cell RNA; the procedure was evaluated by PCR testing 10-fold dilutions of cDNA, synthesised from the extracted RNAs, with bovine and tick specific primers. Although host RNA was not completely removed, RNAse treatment appeared to reduce bovine RNA from endothelial cell preparations as the only signal after PCR was observed with undiluted sample (Fig. 6.5); however, the procedure had little effect on tick cell preparations as a PCR positive signal for tick message was observed down to the 1/1000 dilution (Fig. 6.6). RNA from *E. ruminantium* was reactive in RT-PCR using *E. ruminantium*-specific primers in both preparations.

Fig. 6.3

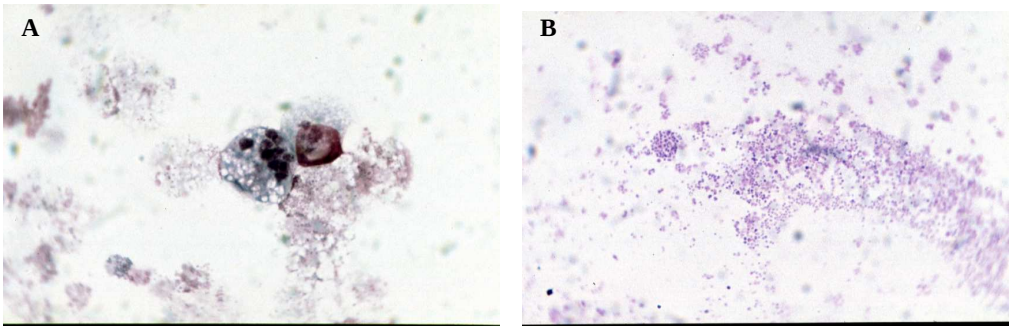


Fig. 6.3: Photomicrographs of Giemsa-stained smears of *E. ruminantium*-infected BPC (A) and mechanically disrupted and Trypsin digested infected cells (B). Magnification x1000 oil immersion.

Fig. 6.4

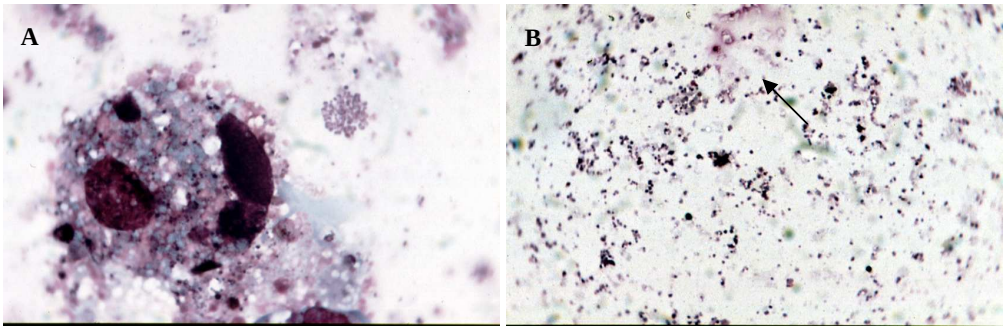


Fig. 6.4: Photomicrographs of Giemsa-stained smears of *E. ruminantium*-infected AVL/CTVM13 (A) and mechanically disrupted and Trypsin digested infected cells (B); arrow points to tick cell debris. Magnification x1000 oil immersion.

Fig. 6.5

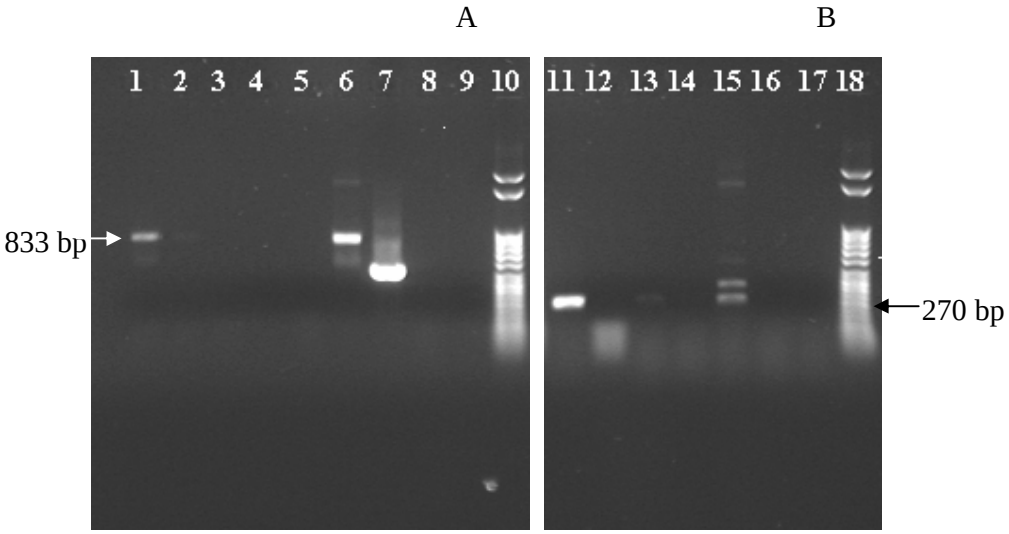


Fig. 6.5: cDNA from dilutions 10^{0} to 10^{-3} (lanes 1-4 and 11-14) of *E. ruminantium*-(Gardel)-infected BPC after purification amplified with *map1* Gfor/Grev (A) and bovine Bov F/R (B) primers. Lane 5: no reverse transcriptase control; lane 6: Gardel gDNA positive control; lane 15: uninfected BPC gDNA; lane 7: RT-PCR positive control; lanes 8,9,16 and 17: no template PCR controls. Lanes 10 and 18: molecular marker.

Fig. 6.6

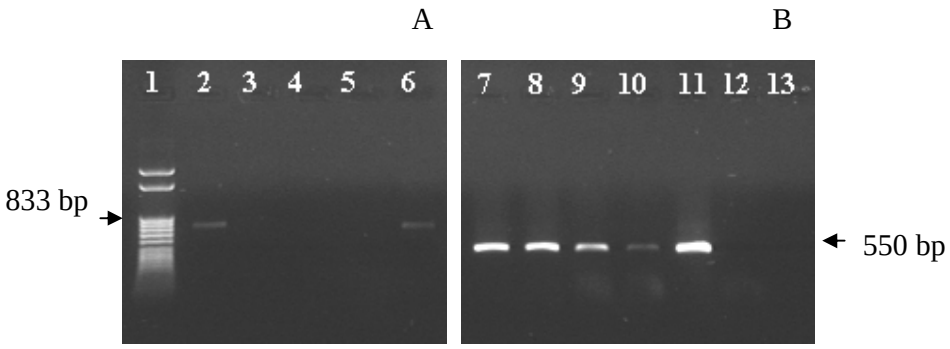


Fig. 6.6: cDNA from dilutions 10^0 to 10^{-3} (lanes 2-5 and 7-10) of *E. ruminantium*-(Gardel)-infected AVL/CTVM13 after purification amplified with *map1* Gfor/Grev (A) and tick Cyt C (B) primers. Lane 6: Gardel gDNA positive control; lane 11: *A. variegatum* gDNA positive control; lane 12 and 13: no template PCR controls. Lane 1: molecular marker.

6.3.3. Separation of bacterial mRNA. In order to remove/reduce bacterial ribosomal RNA, two approaches were tested. First, 16S/23S oligonucleotide-beads in the form of a kit were used; however, no reduction of 23S and very little reduction of 16S *E. ruminantium* ribosomal sequences was observed when the kit was applied to *E. ruminantium* total RNA samples (Figs. 6.7 A, B).

The second approach involved the use of. *E. ruminantium* 16S and 23S rRNA genes amplified by PCR as a second driver during the subtractive step. However, linkers could not been removed from drivers, as verified by successful amplification of the 16S/23S DpnII-digested PCR products with the P24 primer, even after two DpnII digestions of 24h each (Fig. 6.8 A, B). Without good removal of linkers from the driver, enrichment of mRNA could not be achieved and no further steps were taken.

Fig. 6.7A

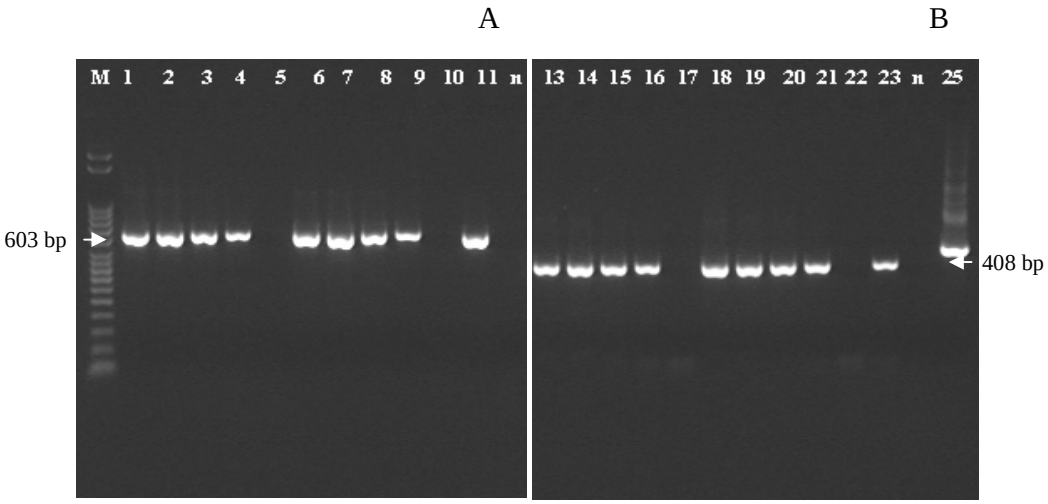


Fig. 6.7A: cDNA from dilutions 10^0 to 10^{-3} (lanes 1-4 *, 6-9 ** and 13-16*, 18-21**) of *E. ruminantium*-infected IDE8, before removal of ribosomal sequences, amplified with 16S (A) and 23S (B) ribosomal specific-primers. Lanes 5, 10, 17 and 22: no reverse transcriptase controls; lane 11 and 23: Gardel gDNA positive controls; lane 25: RT-PCR positive control (523bp); n: no template PCR controls. M: molecular weight marker. (*): sample #1; (**) sample #2.

Fig 6.7B

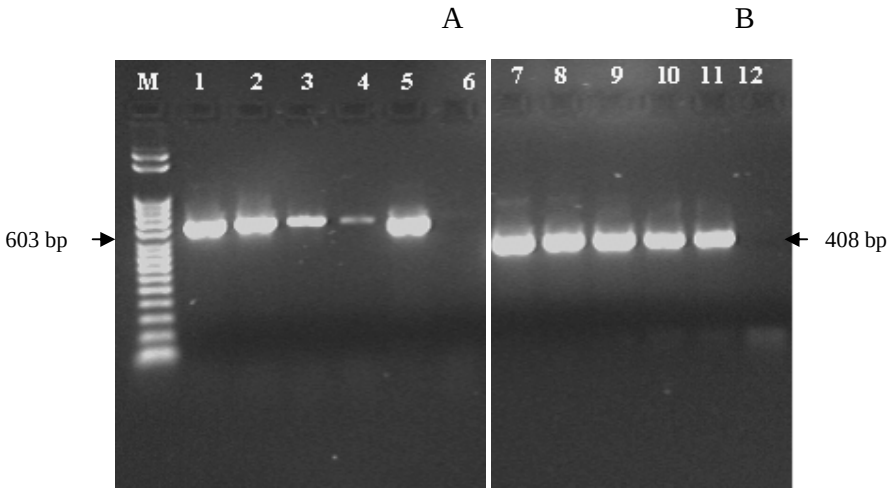


Fig 6.7B: cDNA from dilutions 10^0 to 10^{-3} (lanes 1-4 and 7-10) of *E. ruminantium*-infected IDE8, after removal of ribosomal sequences, amplified with 16S (A) and 23S (B) ribosomal specific-primers. Lanes 5 and 11: Gardel gDNA positive controls; lanes 6 and 12: no template PCR controls. M: molecular weight marker.

Fig. 6.8A

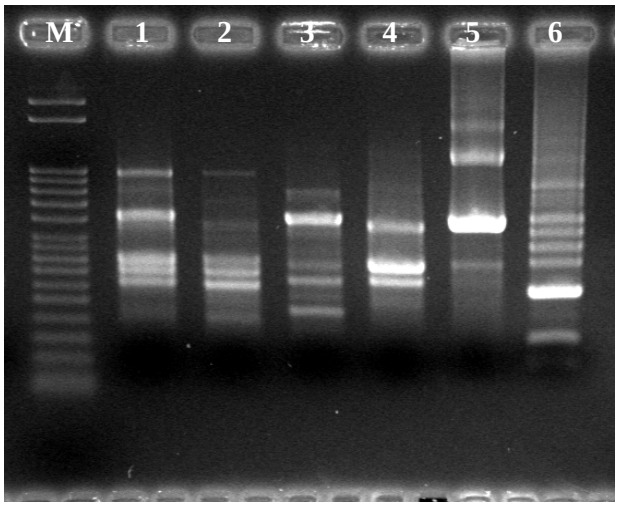


Fig. 6.8A: Testers and drivers before restriction digestion with DpnII. Lanes 1 and 2: linker-ligated PCR products from Gardel growing in AVL/CTVM13 and in BPC cells respectively; lanes 3 and 4: undigested linker-ligated PCR products from uninfected AVL/CTVM13 and BPC respectively; and lanes 5 and 6: linker-ligated PCR products from *E. ruminantium* 16S and 23S rRNA genes. M: 50-bp marker.

Fig. 6.8B

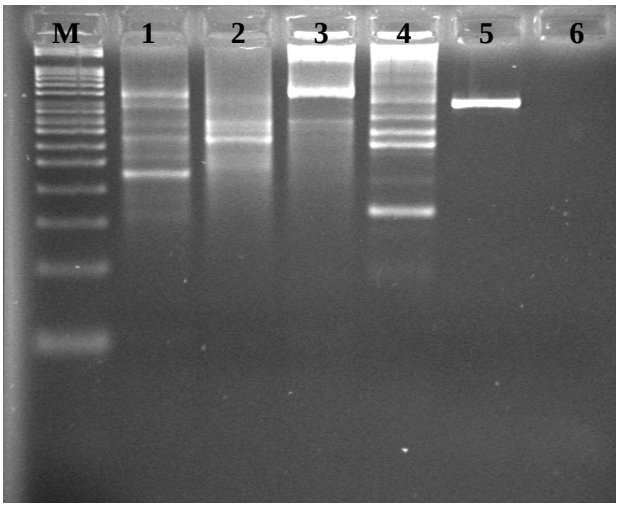


Fig. 6.8B: Drivers after restriction digestion with DpnII. Lanes 1 and 2: digested drivers from uninfected AVL/CTVM13 and BPC respectively; lanes 3 and 4: digested drivers from *E. ruminantium* 16S and 23S rRNA genes. Lane 5: gDNA from uninfected AVL/CTVM13 amplified with tickF/R used as a positive control; lane 6 negative control. M: 50-bp marker.

6.3.4. Suppression subtractive hybridisation (SSH). Resultant products "A", "B" and "C" from the second stage of SSH, were purified, inserted into a vector and transformed into competent *E. coli* cells. Screening of more than a hundred colonies from each product, for inserts between 200-600bp, was carried out by PCR, using primers specific to the vector. Surprisingly, few colonies coming from products "A" and "B" contained inserts higher than 120bp (Figs. 6.9 and 6.10 A,B) (the expected size of the amplified vector alone is 135bp). From product "C", none of the 110 colonies contained an insert higher than 120bp (Figs. 6.9 and 6.10C).

6.3.5. Sequence analysis. Nineteen out of 22 clones analysed gave readable sequences, of which 47% (9) aligned with the 23S ribosomal gene of *E. ruminantium* Welgevonden (CR925678) and Gardel (CR925677) isolates. The remaining sequences did not match with the *E. ruminantium* genome sequence.

Fig. 6.9

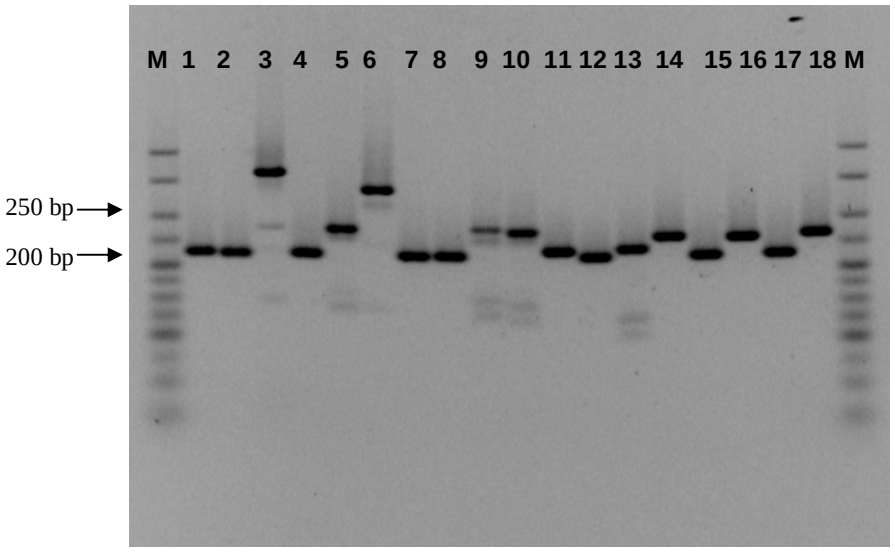


Fig. 6.9: PCR products from positive colonies amplified with primers T7 and SP6: lanes 1-6, colonies # 6, 12, 21, 22, 23 and 25 coming from SSH product “A”; lanes 7-12, colonies # 3, 4, 5, 7, 8 and 11 coming from SSH product “B”; lanes 13-18, colonies # 7, 8, 9a, 9b, 10 and 11 coming from SSH product “C. M: 25-bp marker. 2% metasieve agarose gel stained with ethidium bromide.

Fig. 6.10

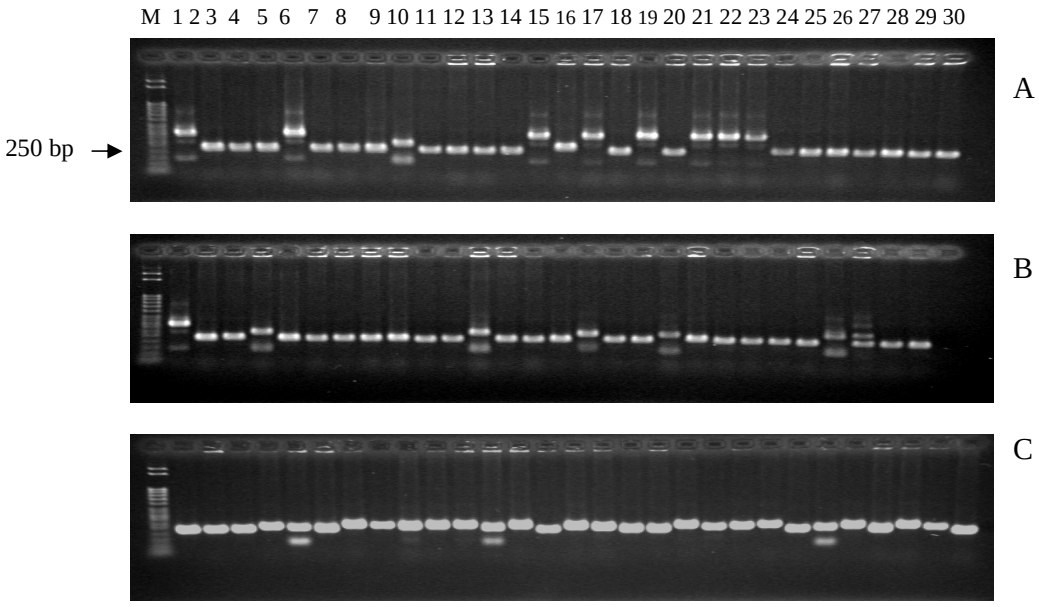


Fig. 6.10: Screening by PCR for inserts higher than 250 bp among white colonies (lanes 1-30). A: white colonies containing SSH "A" inserts amplified with T7 and SP6; B: white colonies containing SSH "B" inserts amplified with T7 and SP6; C: white colonies containing SSH "C" inserts amplified with T7 and SP6. M: 50-bp marker.

6.4 Discussion

The aim of the study presented in this chapter was the identification of genes differentially expressed between *E. ruminantium* (Gardel isolate) grown in endothelial and in tick cell cultures.

One of the major requirements at the outset of this study was to achieve purification of the pathogen from host cells. Since *E. ruminantium* is an obligate intracellular bacterium, it was necessary to apply an efficient and fast purification protocol which would not adversely affect the viability and RNA content of the organism. The extracellular viability of *E. ruminantium* has been demonstrated to decrease enormously within the first 30 minutes at 4°C (L. Bell-Sakyi, personal communication). Therefore, a density gradient centrifugation, such as Percoll, was not considered appropriate for this study since this protocol of purification requires several steps of prolongued centrifugation which would undoubtely affect the final yield of intact RNA. Digestion of cells with trypsin followed by mechanical disruption and low speed centrifugation appeared to clear out most of the host cell debris while maintaining intact ehrlichial organisms. However, as bovine and tick cell transcripts were still detectable in the resultant cDNA preparations, a two step SSH protocol was employed incorporating an initial step to further deplete the cDNA preparations of host cell transcripts. This required addition of linker sequences to the cDNA prepared from both infected cell types, allowing them to be used, in the first step of the procedure, as testers against driver RNAs extracted from the respective

uninfected cells. The linkers then had to be removed from the cDNA preparations used as drivers in the second step of the SSH.

An additional concern was the presence of bacterial ribosomal RNA in the total RNA preparations. The lack of poly (A) tails in prokaryotic mRNA makes it difficult to isolate mRNA free of rRNA. Moreover, since about 85% of the total RNA constitutes rRNA, isolation of total RNA results in a high abundance of rRNA sequences in both tester and driver that could interfere with the procedure, particularly during the subtractive and PCR enrichment steps (Bowler et al., 1999). However, attempts to separate mRNA from total bacterial RNA using 2 different methods were unsuccessful. Although high conservation is observed in the ribosomal sequences of prokaryotic bacteria, it is possible that the failure of the Ambion oligonucleotide-beads to deplete rRNA was due to insufficient specificity in hybridisation with rRNA from ehrlichial pathogens. As an alternative approach, *E. ruminantium* 16S and 23S rRNA obtained by PCR amplification was used as a second driver during the subtractive step (Bowler et al., 1999). However, the failure to excise the linkers from drivers, possibly due to an excessive amount of DNA requiring digestion, led to the abandonment of this method. Ming-Shi et al., 2001, working with *Mycobacterium bovis*, found that hybridisation of single-stranded RNA (driver) with double-stranded (tester) cDNA selectively enriched for differentially expressed sequences and significantly depleted (up to 70%) ribosomal sequences without pre-removal of rRNA. However, large amounts of pure total RNA (at least 50 μg) are required as a driver in this approach; since such amounts could not be obtained from *E. ruminantium* using our purification and extraction protocols, this approach was not feasible. Despite the lack of effective methods for depletion of

rRNA sequences, the cDNA preparations were used in the SSH protocol in the hope that differentially expressed mRNA sequences could still be identified (eg. by size of insert) among contaminating rRNA sequences.

The first stage of SSH, which aimed to reduce contamination of the samples with host cell transcripts, used total RNA extracted from uninfected endothelial and tick cells as drivers to hybridise with linker-ligated cDNA preparations from *E. ruminantium* grown in the same cells as testers. The use of total RNA as a driver has been reported to provide better hybridisation efficiency than a cDNA-cDNA hybridisation since re-annealing of double-stranded driver cDNA is avoided (Ming-Shi et al., 2001). An additional advantage is that the problematical digestion of linkers after PCR amplification to prepare cDNA drivers is avoided. The only point that must be emphasised with this approach is the large amount of total RNA needed; however the quantity of total RNA extracted from uninfected cells in the present studies was not a limiting factor.

In the second stage of the SSH, products resulting from the first stage were used as tester and/or driver in order to find bacterial genes expressed differentially between the two cell types under study, bovine endothelial and tick cells. To prepare the drivers, digestion of linkers was necessary. As mentioned before, this step was very inefficient even after two restriction enzyme digestions of 24h each. Previously, it has been reported that different linkers can be added to the tester and driver in order to avoid the necessity of restriction enzyme digestion of driver (Balzer and Baumlein, 1994). However, Yang and Sytkowski (1996) found that the addition of different linkers gave an unequivalent representation of starting mRNAs for tester and driver, and therefore concluded that it was advantageous to use the same linker.

While they could not completely remove the driver linkers from the driver cDNA by restriction enzyme digestion, they found that the procedure itself has a mechanism to eliminate the residual linkered driver. Thus, if substantial removal of linker is achieved, the unlinkered driver will be present at high excess in the reaction and should drive out the residual linkered driver. Of course, a too high level of linkered driver would still pose a problem for efficiency of enrichment.

More than a hundred cloned products obtained from the second stage of SSH, in 3 experiments, were screened in an attempt to identify inserts between 200-600 bp. Surprisingly, few of the *E. coli* colonies coming from products "A" and "B" contained inserts higher than 120 bp (Figs. 6.9 and 6.10 A, B) (the expected size of the amplified vector alone is 135 bp). From product "C", none of the 110 colonies contained an insert higher than 120 bp (Figs. 6.9 and 6.10C). The abundance of small fragments could not have been due to the restriction enzyme chosen for the process, DpnII; this enzyme was selected because it gives fragments between 100-800bp, as confirmed against the current full genome sequence of the Gardel isolate (Frutos et al., 2006), that are favoured by PCR amplification (Bowler et al., 1999). However, it is possible that the smallest fragments were preferentially enriched by the SSH protocol.

Finally, 22 inserts of greater than 120 bp were selected at random for sequencing. Comparison of the resultant sequences with the PubMed database (when the full sequences of the Welgevonden and Gardel isolate genomes were available) revealed that the final cDNA populations were substantially contaminated with *E. ruminantium* rRNA sequences. Approximately 50% of the inserts analysed aligned to the 23S ribosomal gene of *E. ruminantium* Welgevonden and Gardel isolates. The

remaining sequences did not match with anything in the *E. ruminantium* genome and presumably represented fragments of host cell transcripts.

In summary, differentially expressed *E. ruminantium* transcripts were not sufficiently represented in the final cDNAs to allow their detection. This was due to substantial contamination with rRNA and probably residual host cell transcripts. The difficulty experienced in removing the linkers was likely to have been a major factor in hindering the subtraction of the sequences common to the two populations. Therefore, an optimisation of the ratio of PCR product to amount of restriction enzyme, and incubation periods, must be considered in any future attempts in order to achieve a successful removal of linkers. However, the availability of the full *E. ruminantium* genome sequences (which were not available at the outset of these studies) now allows alternative, more straightforward approaches utilising microarrays to identify differentially expressed genes.

Chapter 7: General discussion

The economic importance of heartwater as a constraint to livestock improvement programmes in sub-Saharan Africa and the Caribbean (Camus et al., 1996; Mukhebi et al., 1999) and the current lack of effective and affordable vaccines (Camus et al., 1996; Mahan et al., 1999; Collins et al., 2003) has been the main driving force behind recent research into the disease. Progress in this regard requires a better understanding of the kinetics of infection in the mammalian host and also information on expression of immunogenic antigens during different stages of development of the organism.

The work presented in this thesis has contributed to several areas of importance, namely quantification of *E. ruminantium* organisms in ticks, identification of *map1* genes differentially transcribed *in vivo* in different tissues of infected *A. variegatum* ticks and the antigenic characterisation of proteins encoded by these genes.

Studies on gene and protein expression of *E. ruminantium* in the mammalian host have been hindered by the difficulty of detecting these organisms by PCR in circulating blood during both acute and persistent (carrier) infections. Accordingly, a quantitative real-time PCR method, considered as likely to be more sensitive than conventional PCR and DNA hybridisation methods, was standardised to study the kinetics of infection in sheep inoculated with mammalian cell-derived *E. ruminantium*. Because of the difficulty experienced in obtaining accurate counts of *E. ruminantium* organisms by microscopy, the generation of a calibration curve based on data from dilutions of the bacteria proved unreliable. A standard curve based on dilutions of plasmids encoding the *map1-1* gene was therefore used and this

was found to improve the reproducibility and accuracy of the quantification (Chapter 2). By reference to this standard curve, it was estimated that the system was able to detect as few as 100 organisms/µl blood. *E. ruminantium*, however, was only detected in infected sheep during the febrile phase of the infection, indicating that the organisms were present at low concentrations in peripheral blood during the pre-clinical phase (Postigo et al., 2002; Chapter 2). Kock et al. (1995) reported that DNA of *E. ruminantium* could be amplified in some instances from bone marrow, but not from whole blood samples (1 ml) using the *map1* gene as a PCR target. In contrast Mahan et al. (1992), using DNA probe hybridisations, was able to detect *E. ruminantium* in 5-10 ml samples of plasma from sheep before the onset of fever. In the present study, small volumes of whole blood (200 µl) were used, of which only 1% was used in each PCR assay. However, the use of pellets of white cells collected from 5 ml of blood did not increase the sensitivity of detection, suggesting that the organisms were present in other fractions of the blood, possibly the serum. Collectively, these observations suggest that assays suitable for testing larger sample volumes of blood, including enrichment of the appropriate blood fractions, need to be used to successfully investigate the dynamics of *E. ruminantium* in infected mammals. Nevertheless, the real-time PCR method developed in the present study made it possible, for the first time, to detect and provide relative quantitative data on the presence of *E. ruminantium* in different tissues of ticks during transmission to naïve sheep, thus helping to elucidate the nature and location of developmental stages of the pathogen in the tick vector. The technique could also prove to be useful to detect *E. ruminantium* in field-collected ticks for ecological studies and epidemiological surveillance of heartwater.

The availability of *in vitro* culture systems in both mammalian and tick cells, and access to a laboratory tick colony, provided an opportunity for the present project to examine the expression of *E. ruminantium* genes in the different hosts. It was reasoned that adaptation of the pathogen to host and vector might be reflected in molecular and biological differences in organisms grown in different *in vitro* systems. Application of the SSH technique to RNA was a novel approach which, although not resulting in identification of any differentially transcribed genes in the present study, was shown to work in principle. The major problem encountered, which could not be resolved within the time available for the project, was the difficulty of removing bacterial ribosomal RNA from the test samples. Although there are several approaches that could be taken to overcome this problem, alternative methods based on microarrays, utilising the full genome sequences for *E. ruminantium* (which were not available when these experiments were carried out), would be more appropriate to address this question in future studies.

The research then focused on the *map1* multigene family. Data from previous studies utilising MAP-specific reverse transcriptase PCRs (Bekker et al., 2002) and Western blots with monoclonal antibodies against MAP1 (S. Smith, personal communication), suggested that *E. ruminantium map* paralog genes are differentially expressed in organisms grown in tick and the mammalian culture systems. Several molecular techniques including the RT-PCR assay referred to above, and proteomics using 2DE, were used to address three questions. First, which MAP proteins are differentially expressed by organisms grown in different host cells *in vitro*? Second: which MAP proteins are expressed *in vivo* in the tick and in which tissue? Third,

does expression of the MAP proteins change during tick feeding and is expression of particular proteins associated with acquisition of infectivity?

The lack of pathogenicity of *E. ruminantium* grown in tick cells *in vitro* and, in some cases, infectivity for sheep (Bell-Sakyi et al., 2002), supports the hypothesis that the stage of development present in infected tick cell cultures is not the same as that transmitted during feeding by an infected tick, which results in a severe clinical disease. Rather, these cultured organisms may be similar to the stage which multiplies in tick midguts after ingestion of an infected blood meal. Moreover, *E. ruminantium* is not transmitted by infected *Amblyomma* ticks until the tick has been feeding for 1-4 days (Camus et al., 1996) suggesting that the pathogen requires a period of development before it becomes infective for the mammalian host. However, when RT-PCR analyses were carried out, no obvious differences were observed in transcription of members of the *map1* cluster between *E. ruminantium* grown *in vitro* in bovine endothelial cells, which are highly infective for sheep, and those grown in tick cells, which have low or no infectivity for sheep. Care must be taken in interpreting these results since the patterns of transcription *in vitro* do not necessarily reflect the activity of organisms *in vivo*. As an example, studies with *E. canis* have shown all 22 *p28* paralogs were transcribed in a canine monocyte cell line (DH82) whereas only 11 paralogs were detected in infected dogs (Ohashi et al., 2001; Unver et al., 2001).

Transmission trials were therefore carried out in order to examine the patterns of transcription of genes of the *map1* multigene family in the tissues of infected ticks before and during transmission feeding. The results of these experiments demonstrated differential expression of these genes in midgut and

salivary glands, with markedly increased levels of transcription of the *map1-1* gene in midguts and of the *map1* gene in salivary glands. Comparison with the numbers of bacteria present in the different tick tissues during feeding indicates that these results reflect an upregulation of these genes within the respective tissues and not merely an increase in the number of bacteria. Data from real-time PCR confirmed that *E. ruminantium* was already present in both midguts and salivary glands of infected ticks before the start of the transmission feeding and that the number of bacteria increased substantially after 2 days of feeding in both tissues, but decreased dramatically in salivary glands between days 3 and 4. This latter period coincided with the time when the feeding *A. variegatum* ticks would be expected to be transmitting *E. ruminantium* (Camus et al., 1996), suggesting that the reduced quantities in salivary glands was due to release into the saliva.

To determine whether the differential transcription of *map1* paralogs observed *in vivo* is reflected at the protein level, a proteomics approach was followed. As a first step, because of the tiny quantities of these proteins in tissues from infected ticks and ruminants, the presence of host cell-specific *E. ruminantium* proteins encoded by the *map1* cluster was investigated using organisms grown *in vitro* in tick cells and bovine endothelial cells. 2DE gels and MALDI-TOF MS analysis of the most prominent spots of around 30 kDa revealed that the immunodominant protein MAP1 was abundant in organisms grown in endothelial cells, whereas MAP1-1 protein was abundant in *E. ruminantium* grown in tick cells. Additional analysis provided evidence that these proteins are glycosylated. Since transcripts for both genes were detected in both cell types but at the protein level MAP1 predominated in endothelial cells while MAP1-1 predominated in tick cells,

this would suggest either that *map1* and *map1-1* transcripts are inefficiently translated in the tick and endothelial cell lines respectively or, more likely, that the proteins are expressed at lower levels, or not at all, in these cell types.

The detection of higher levels of *map1* transcripts in salivary glands than in midguts of infected ticks, together with the presence of abundant MAP1 protein in organisms grown in mammalian but not in tick cell lines, suggest that expression of this protein may be associated with infectivity for mammalian cells. It is possible that efficient expression of MAP1 protein within the tick is dependent on growth within a differentiated cell type in the salivary gland, which is likely to be absent from the embryo-derived cell lines. Analysis of protein expression in the present study focused only on the most abundant protein spots. Further studies are required to investigate other protein spots that differ between infected tick and mammalian cells including other members of the MAP1 family. The proteomic method employed in the present study will also be useful, in conjunction with other methodologies, for investigation of other biologically relevant *E. ruminantium*/host cell interactions, including alteration of host cell protein expression by infection with *E. ruminantium*, and understanding the basis of attenuation by comparison of both host and bacterial proteomes in cells infected with virulent and attenuated isolates of *E. ruminantium*.

Together the data generated by the present study indicate that: i) the tick midgut is the main tissue site for colonisation and replication of *E. ruminantium* as the number of bacteria always remained higher in midguts than in salivary glands; ii) the organisms replicating in the salivary glands, and transcribing the *map1* gene, are the infective form of *E. ruminantium*; iii) the saliva is likely to be the principal

vehicle for transmission of *E. ruminantium*; iv) differential expression of *E. ruminantium map1* gene family paralogs in unfed and feeding ticks reflects differentiation from vegetative to mammalian-infective forms during transmission feeding; v) the MAP1-1 protein may have an important function in early development within the vector, since *map1-1* transcripts were abundant both in midguts of infected ticks and in tick cell lines, and the protein was expressed at high levels in infected tick cell cultures. In relation to this last point, identification and functional characterisation of the *A. variegatum* tick ligands that interact with MAP1-1 would advance our understanding of the role of this protein in development within the tick.

The Osp-A-based Lyme disease vaccine prevents transmission of *B. burgdorferi* to mammals by neutralising spirochaetes in the tick gut after the tick has taken a blood meal (de Silva et al., 1996; Pal et al., 2001). Similarly, anti OspB antibodies which bind to a protein or proteins within the tick gut interfere with *B. burgdorferi* colonisation of *I. scapularis* (Fikrig et al., 2004). By analogy, the MAP1-1 protein might be a vaccine candidate for the prevention of *E. ruminantium* transmission from ticks to the mammalian host by preventing efficient colonisation of the vector, thereby interfering with the life cycle of *E. ruminantium*.

References

Alberdi M. P., A. R. Walker, E. A. Paxton & K. J. Sumption (1998). Natural prevalence of infection with *Ehrlichia* (*Cytoecetes*) *phagocytophila* of *Ixodes ricinus* ticks in Scotland. *Vet. Parasitol.* 78: 203-213.

Allsopp, M. T., C. M. Hattingh, S. W. Vogel & B. A. Allsopp (1998). Comparative evaluation of 16S, map1 and pCS20 probes for the detection of *Cowdria* and *Ehrlichia* species in ticks. *Ann. N Y Acad. Sci.* 29: 78-84.

Allsopp, M. T. E. P., C. M. Dorfling, J. C. Maillard, A. Bensaid, D.T. Haydon, H. van Heerden & B. A. Allsopp (2001). *Ehrlichia ruminantium* major antigenic protein gene (*map1*) variants are not geographically constrained and show no evidence of having evolved under positive selection pressure. *J. Clin. Microbiol.* 39: 4200-4203.

Andrew, H. R. & R. A. I. Norval (1989). The carrier status of sheep, cattle and African buffalo recovered from heartwater. *Vet. Parasitol.* 34: 261-266.

Anon (1997). Development and testing of thermostable immunogens for the protection of ruminants against heartwater. DfID Animal Health Programme Project N^{o} R6566, Final Technical Report, Centre for Tropical Veterinary Medicine, University of Edinburgh, Scotland.

Asselbergs, M., F. Jongejan, A. Langa, L. Neves & S. Afonso (1993). Antibodies to *Cowdria ruminantium* in Mozambican goats and cattle detected by immunofluorescence using endothelial cell culture antigen. *Trop. Anim. Hlth Prod.* 25: 144-150.

Balzer, H. J. & H. Bäumlein (1994). An improved gene expression screen. *Nucleic Acids Res* 22: 2853-2854.

Barbet, A. F., A. Lundgren, J. Yi, F. R. Rurangirwa & G. H. Palmer (2000). Antigenic variation of *Anaplasma marginale* by expression of MSP2 mosaics. *Infect. Immun.* 68: 6133-6138.

Barbet, A. F., S. M. Semu, N. Chigagure, P. J. Kelly, F. Jongejan & S. M. Mahan (1994). Size variation of the major immunodominant protein of *Cowdria ruminantium*. *Clin. Diagn. Lab. Immunol.* 1: 744-746.

Barbet, A. F., J. Yi, A. Lundgren, B. R. McEwen, E. F. Blouin & K. M. Kocan (2001). Antigenic variation of *Anaplasma marginale*: major surface protein 2 diversity during cyclic transmission between ticks and cattle. *Infect. Immun.* 69: 3057-3066.

Barbet, A. F., J. Agnes, A. Moreland, A. Lundgren, A. R. Alleman, S. Noh, K. A. Brayton, U. G. Munderloh & G. H. Palmer (2005). Identification of functional promoters in the *msp2* expression loci of *Anaplasma marginale* and *Anaplasma phagocytophilum. Gene* 353: 89-97.

Barré, N., G. Uilenberg, P. C. Morel & E. Camus (1987). Danger of introducing heartwater onto the American mainland: potential role of indigenous and exotic *Amblyomma* ticks. *Onderstepoort J. Vet. Res.* 54: 405-417.

Bekker, C. P. J., L. Bell-Sakyi, E. A. Paxton, D. Martinez, A. Bensaid & F. Jongejan (2002). Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*. *Gene* 285: 193-201.

Bekker, C. P. J., M. Postigo, A. Taoufik, L. Bell-Sakyi, C. Ferraz, D. Martinez & F. Jongejan (2005). Transcription analysis of the major antigenic protein 1 multigene family of three *in vitro*-cultured *Ehrlichia ruminantium* isolates. *J. Bacteriol.* 187: 4782-4791.

Belland, R. J., G. Zong, D. D. Crane, D. Hogan, D. Sturdevant, J. Sharma, W. L. Beatty & H. D. Caldwell (2003). Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis. Proc. Natl. Acad. Sci. U.S.A.* 100: 8040-8042.

Bell-Sakyi, L. (2004). Epidemiology of heartwater in Ghana and growth of *Ehrlichia ruminantium* in tick cell lines. PhD thesis. Utrecht University, 205 pp.

Bell-Sakyi, L., E. A. Paxton, U. G. Munderloh & K. J. Sumption (2000a). Growth of *Cowdria ruminantium*, the causative agent of heartwater, in a tick cell line. *J. Clin. Microbiol.* 38: 1238-1240.

Bell-Sakyi, L., E. A. Paxton, U. G. Munderloh & K. J. Sumption (2000b). Morphology of *Cowdria ruminantium* grown in two tick cell lines. In: Proceedings of the 3rd International Conference "Tick and Tick-borne Pathogens: Into the 21st Century", M. Kazimirova, M. Labuda, P. A. Nuttall (eds), Institute of Zoology, Slovak Academy of Sciences , Bratislava, Slovakia, pp131-137.

Bell-Sakyi, L., E. A. Paxton, P. Wright & K. J. Sumption (2002). Immunogenicity of *Ehrlichia ruminantium* grown in tick cell lines. *Exp. Appl. Acarol.* 28: 177-185.

Bell-Sakyi, L., E. B. M. Koney, O. Dogbey, K. J. Sumption, A. R. Walker, A. Bath & F. Jongejan (2003). Detection by two enzyme-linked immunosorbent assays of antibodies to *Ehrlichia ruminantium* in field sera collected from sheep and cattle in Ghana. *Clin. Diagn. Lab. Immunol.* 10: 917-925.

Bezuidenhout, J. D. (1981). The development of a new heartwater vaccine using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. In: “Tick Biology and Control”, G. B. Whitehead, J. D Gibson (eds), Tick Research Unit, Grahamstown, South Africa, pp41-45.

Bezuidenhout, J. D. (1987). Natural transmission of heartwater. *Onderstepoort J. Vet. Res.* 56: 349-351.

Bezuidenhout, J. D. (1988). Certain aspects of the transmission of heartwater, the occurrence of the organism in ticks and *in vitro* culture. DVSc Thesis. University of Pretoria, South Africa.

Bezuidenhout, J. D. & C. J. Jacobsz (1986). Proof of transovarial transmission of *Cowdria ruminantium* by A*mblyomma hebraeum*. *Onderstepoort J. Vet. Res.* 53: 31-34.

Bezuidenhout, J. D., C. L. Paterson & B. J. H. Barnard (1985). *In vitro* cultivation of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 52: 113-120.

Bezuidenhout, J. D., J. A. Olivier, B. Gruss & J. V. Badenhorst (1987). The efficacy of alternative routes for the infection or vaccination of animals with *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 54: 497-506.

Blouin, E. F., J. T. Saliki, J. de la Fuente, J. C. Garcia-Garcia & K. M. Kocan (2003). Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infectivity for cultured tick cells. *Vet. Parasitol.* 111: 247-260.

Bowie, M. V., G. R. Reddy, S. M. Semu, S. M. Mahan & A. F. Barbet (1999). Potential value of major antigenic protein 2 for serological diagnosis of heartwater and related ehrlichial infections. *Clin. Diagn. Lab. Immunol.* 6: 209-215.

Bowler, L.D., M. Hubank & B. G. Spratt (1999). Representational difference analysis of cDNA for the detection of differential gene expression in bacteria: development using a model of iron-regulated gene expression in *Neisseria meningitidis*. *Microbiology* 145: 3529-3537.

Brayton, K. A., D. P. Knowles, T. C. McGuire & G. H. Palmer (2001). Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 98: 4130-4135.

Brayton, K. A., P. F. M. Meeus, A. F. Barbet & G. H. Palmer (2003). Simultaneous variation of the immunodominant outer membrane proteins, MSP2 and MSP3, during *Anaplasma marginale* persistence *in vivo*. *Infect. Immun.* 71: 6627-6632.

Brayton, K. A., J. Fehrsen, E. P. De Villiers, M. van Kleef & B. A. Allsopp (1997). Construction and initial analysis of a representative lambda ZAPII expression library of the intracellular rickettsia *Cowdria ruminantium*: cloning of *map1* and three other *Cowdria* genes. *Vet. Parasitol.* 72: 185-199.

Brayton, K. A., G. H. Palmer, A. Lundgren, J. Yi & A. F. Barbet (2002). Antigenic variation of *Anaplasma marginale msp2* occurs by combinatorial gene conversion. *Mol. Microbiol.* 43: 1151-1159.

Brayton, K. A., L. S. Kappmeyer, D. R. Herndon, M. J. Dark, D. L. Tibbals, G. H. Palmer, T. C. McGuire & G. P. J. Knowles (2005). Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.*102(3): 844-9.

Caldwell, H. D., J. Kromhout & J. Schatcher (1981). Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* 31: 1161-1176.

Camus, E., N. Barre, D. Martinez & G. Uilenberg (1996). Heartwater (cowdriosis) a review. 2nd edition. O.I.E., Paris, 177pp.

Camus, E. & N. Barré (1982). La cowdriose (heartwater). Revue Génerale des Connaissances, Institut d' Élevage et Médicine Vétérinaire des Pays Tropicaux, Maisons-Alfort, 147pp.

Carlyon, J. A. & E. Fikrig (2003). Invasion and survival strategies of *Anaplasma phagocytophilum*. *Cell Microbiol.* 5: 743-754.

Cheng, C., C. D. Paddock & R. Reddy Ganta (2003). Molecular heterogeneity of *Ehrlichia chaffeensis* isolates determined by sequence analysis of the 28-kilodalton outer membrane protein genes and other regions of the genome. *Infect. Immun.* 71: 187-195.

Collins, N. E., A. Pretorius, M. van Kleef, K. A. Brayton, E. Zweygarth & B. Allsopp (2003). Development of improved vaccines for heartwater. *Ann. N Y Acad. Sci.* 990: 474-484.

Collins, N. E., J. Liebenberg, E. P. De Villiers, K. A. Brayton, E. Louw, A. Pretorius, E. Faber, H. van Heerden, A. I. Josemans, M. van Kleef, H. C. Steyn, F. van Strijp, E. Zweygarth, F. Jongejan, J. C. Maillard, D. Berthier, M. Botha, F. Joubert, C. Corton, N. Thomson, M. T. Allsopp & B. A. Allsopp (2005). The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proc. Natl. Acad. Sci. U.S.A.* 102: 838-843.

Cowdry, E. V. (1925a). Studies on the aetiology of heartwater. I. Observation of a rickettsia, *Rickettsia ruminantium* (n.sp.) in the tissues of infected animals. *J. Exp. Med.* 42: 231-252.

Cowdry, E. V. (1925b). Studies on the aetiology of heartwater. II. *Rickettsia ruminantium* (n. sp.) in the tissues of ticks transmitting the disease. *J. Exp. Med.* 42: 253-274.

Cowdry, E. V. (1926). Studies on the aetiology of heartwater: III. The multiplication of *Rickettsia ruminantium* within the endothelial cells of infected animals and their discharge into the circulation. *J. Exp. Med.* 44: 803-814.

De Silva, A. M., S. M. Telford 3rd, L.R. Brunet, S.W. Barthold & E. Fikrig (1996). *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *J. Exp. Med.* 183: 271-275.

Diatchenko, L., Y. F. Lau, A. P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E. D. Sverdlov & P. D. Siebert (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. U.S.A.* 93: 6025-6030.

Dixon, R. (1898). Heartwater experiments. *Agric. J. Cape of Good Hope* 12: 754-762.

Du Plessis, J. L. (1970). Pathogenesis of heartwater: I. *Cowdria ruminantium* in the lymph nodes of domestic ruminants. *Onderstepoort J. Vet. Res.* 37: 89-96.

Du Plessis, J. L. (1975). Electron microscopy of *Cowdria ruminantium* infected reticulo-endothelial cells of the mammalian host. *Onderstepoort J. Vet. Res.* 42: 1-14.

Du Plessis, J. L. & L. Malan (1987). The non-specific resistance of cattle to heartwater. *Onderstepoort J. Vet. Res.* 54: 333-336.

Du Plessis, J. L., L. van Gas, J. Olivier & J. D. Bezuidenhout (1989). The heterogenicity of *Cowdria ruminantium* stocks: cross immunity and serology in sheep and pathogenicity to mice. *Onderstepoort J. Vet. Res.* 56: 195-201.

Dumler, J. S., A. F. Barbet, C. P. J. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa & F. R. Rurangirwa (2001). Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma, Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Microbiol.* 51: 2145-2165.

Esteves, I., N. Vachiery, D. Martinez & P. Totté (2004). Analysis of *Ehrlichia ruminantium*-specific T1/T2 responses during vaccination with a protective killed vaccine and challenge of goats. *Parasite Immunol.* 26: 95-103.

Felek, S., R. Greene & Y. Rikihisa (2003). Transcriptional analysis of p30 major outer membrane protein genes of *Ehrlichia canis* in naturally infected ticks and sequence analysis of *p30-10* of *E. canis* from diverse geographic regions. *J. Clin. Microbiol.* 41: 886-888.

Fikrig, E., U. Pal, C. Manchuan, J.F. Anderson & R. A. Flavell (2004). OspB antibody prevents *Borrelia burgdorferi* colonization of *Ixodes scapularis*. *Infect. Immun.* 72: 1755-1759.

Foster, J., M. Ganatra, I. Kamal, J. Ware, K. Makarova, N. Ivanova, A. Bhattacharyya, V. Kapatral, S. Kumar, J. Posfai, T. Vincze, J. Ingram, L. Moran, A. Lapidus, M. Omelchenko, N. Kyrpides, E. Ghedin, S. Wang, E. Goltsman, V. Joukov, O. Ostrovskaya, K. Tsukerman, M. Mazur, D. Comb, E. Koonin & B. Slatko (2005). The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PloS Genet.* 3: e121.

French, D. M., W. C. Brown & G. H. Palmer (1999). Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. *Infect. Immun.* 67: 5834-5840.

French, D. M., T. F. McElwain, T. C. McGuire & G. H. Palmer (1998). Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect. Immun.* 66: 1200-1207.

Frutos, R., A. Viari, C. Ferraz, A. Morgat, S. Eychenié, Y. Kandassamy, I. Chantal, A. Bensaid, E. Coissac, N. Vachiery, J. Demaille & D. Martinez (2006). Comparative genomic analysis of three strains of *Ehrlichia ruminantium* reveals an active process of genome size plasticity. *J. Bacteriol.* 188: 2533-42.

Futse, J., K. A. Brayton, D. P. Knowles & G. H. Palmer (2005). Structural basis for segmental gene conversion in generation of *Anaplasma marginale* outer membrane protein variants. *Mol. Microbiol.* 57: 212-221.

Garcia-Canas, V., R. Gonzales & A. Cifuentes (2002). Ultrasensitive detection of genetically modified maize DNA by capillary gel electrophoresis with laser-induced fluorescence using different fluorescent intercalating dyes. *J. Agric. Food Chem.* 50: 4497-4502.

Garcia-Garcia, J. C., J. de la Fuente, G. Bell-Eunice, E. F. Blouin & K. M. Kocan (2004). Glycosylation of *Anaplasma marginale* major surface protein 1a and its putative role in adhesion to tick cells. *Infect. Immun.* 72: 3022-3030.

Gueye, A., F. Jongejan, M. Mbengue, A. Diouf & G. Uilenberg (1994). Essai sur le terrain d'un vaccin atténué contre la cowdriose. *Rev .Élev. Méd. vét. Pays trop.* 47: 401-404.

Hart, A., K. M. Kocan, J. D. Bezuidenhout & L. Prozesky (1991). Ultrastructural morphology of *Cowdria ruminantium* in midgut epithelial cells of adult *Amblyomma hebraeum* female ticks. *Onderstepoort J. Vet. Res.* 58: 187-193.

Hatch, T. P., I. Allan & J. H. Pierce (1984). Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. *J. Bacteriol.* 157: 13-20.

Heyne, W., E. G. Elliot & J. D. Bezuidenhout (1987). Rearing and infection techniques for *Amblyomma* species to be used in heartwater transmission experiments. *Onderstepoort J. Vet. Res.* 54: 461-471.

Hodzic E., F. Durland, C. M. Maretzki, A. M. De Silva, S. Feng & S. W. Barthold (1998). Acquisition and transmission of the agent of human granulocytic ehrlichiosis by *Ixodes scapularis* ticks. *J. Clin. Microbiol.* 36:3574-3578

Hotopp, J. C., M. Lin, R. Madupu, J. Crabtree, S. V. Angiuoli, J. Eisen, R. Seshadri, Q. Ren, M. Wu, T. Utterback, S. Smith, M. Lewis, C. Zhang, H. Niu, Q. Lin, N. Ohashi, N. Zhi, W. Nelson, L. M. Brinkac, R. Dodson, R. M.J., J. Sundaram, S. C. Daugherty, T. Davidsen, A. Durkin, M. Gwinn, D. H. Haft, J. D. Selengut, S. A. Sullivan, N. Zafar, L. Zhou, F. Benahmed, H. Forberger, R. Halpin, S. Mulligan, J. Robinson, O. White, Y. Rikihisa & H. Tettelin (2006). Comparative genomics of emerging human ehrlichiosis agents. *PloS Genet.* 2(2): e21.

Hutcheon, D. (1900). The history of heartwater. *Agric. J. Cape of Good Hope* 17: 410-417.

IJdo, J., C. Wu, S. R. Telford III & E. Fikrig (2002). Differential expression of the *p44* gene family in the agent of human granulocytic ehrlichiosis. *Infect. Immun.* 70: 5295-5298.

Jongejan, F. (1991). Protective immunity to heartwater (*Cowdria ruminantium* infection) is acquired after vaccination with *in vitro*-attenuated rickettsiae. *Infect. Immun.* 59: 729-731.

Jongejan, F. & M. J. C. Thielemans (1989). Identification of an immunodominant antigenically conserved 32-kilodalton protein from *Cowdria ruminantium*. *Infect. Immun.* 57: 3243-3246.

Jongejan, F., M. J. C. Thielemans, C. Brière & G. Uilenberg (1991a). Antigenic diversity of *Cowdria ruminantium* isolates determined by cross-immunity. *Res. Vet. Sci.* 51: 24-28.

Jongejan, F., M. J. C. Thielemans, M. de Groot, P. J. van Kooten & B. A. M. van der Zeijst (1991b). Competitive enzyme-linked immunosorbent assay for heartwater using monoclonal antibodies to a *Cowdria ruminantium*-specific 32- kilodalton protein. *Vet. Microbiol.* 28: 199-211.

Jongejan, F., N. de Vries, J. Nieuwenhuijs, A. H. M. van Vliet & L. A. Wassink (1993). The immunodominant 32-kilodalton protein of *Cowdria ruminantium* is conserved within the genus *Ehrlichia*. *Rev. Élev. Méd. vét. Pays trop.* 46: 145-152.

Jongejan, F., L. A. Wassink, M. J. C. Thielemans, M. Perie & G. Uilenberg (1989). Serotypes in *Cowdria ruminantium* and relationship with *Ehrlichia phagocytophila* determined by immunofluorescence. *Vet. Microbiol.* 21: 31-40.

Jongejan, F., T. A. Zandbergen, P. A. van de Wiel, M. de Groot & G. Uilenberg (1991c). The tick-borne rickettsia *Cowdria ruminantium* has a *Chlamydia*-like developmental cycle. *Onderstepoort J. Vet. Res.* 58: 227-237.

Kocan, K. M. & J. D. Bezuidenhout (1987). Morphology and development of *Cowdria ruminantium* in *Amblyomma* ticks. *Onderstepoort J. Vet. Res.* 54: 177-182.

Kocan, K. M., J. D. Bezuidenhout & A. Hart (1987a). Ultrastructural features of *Cowdria ruminantium* in midgut epithelial cells and salivary glands of nymphal *Amblyomma hebraeum*. *Onderstepoort J. Vet. Res.* 54: 87-93.

Kocan, K. M., S. P. Morzaria, W. P. Voigt, J. Kiarie & A. D. Irvin (1987b). Demonstration of colonies of *Cowdria ruminantium* in midgut epithelial cells of *Amblyomma variegatum*. *Am. J. Vet. Res.* 48: 356-360.

Kock, N. D., A. H. M. van Vliet, K. Charlton & F. Jongejan (1995). Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. *J. Clin. Microbiol.* 33: 2501-2504.

Li, M. S., I. M. Monahan, S. J. Waddell, J. A. Mangan, S. L. Martin, M. J. Everett & P. D. Butcher (2001). cDNA-RNA subtractive hybridization reveals increased expression of mycocerosic acid synthase in intracellular *Mycobacterium bovis* BCG. *Microbiology* 147: 2293-2305.

Lin, M., Y. Rikihisa, S. Felek, X. Wang, R. Massung & Z. Woldehiwet (2004). *Anaplasma phagocytophilum* has a functional *msp2* gene that is distinct from *p44. Infect. Immun.* 72: 3883-3889.

Logan, L. L., T. C. Whyard, J. C. Quintero & C. A. Mebus (1987). The development of *Cowdria ruminantium* in neutrophils. *Onderstepoort J. Vet. Res.* 54: 197-204.

Löhr, C. V., K. A. Brayton, A. F. Barbet & G. H. Palmer (2004). Characterization of the *Anaplasma marginale msp2* locus and its synteny with the *omp1*/*p30* loci of *Ehrlichia chaffeensis* and *E. canis*. *Gene* 325: 115-121.

Löhr, C. V., F. R. Rurangirwa, T. F. McElwain, D. Stiller & G. H. Palmer (2002). Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. *Infect. Immun.* 70: 114-120.

Long, S. W., X. F. Zhang, H. Qi, S. Standaert, D. H. Walker & X. J. Yu (2002). Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. *Infect. Immun.* 70: 1824-1831.

Lounsbury, C. (1900). Tick heartwater experiments. *Agric. J. Cape of Good Hope* 16: 682-687.

Mahan, S. M., B. H. Simbi & M. J. Burridge (2004). The pCS20 PCR assay for *Ehrlichia ruminantium* does not cross-react with the novel deer ehrlichial agent found in white-tailed deer in the United States of America. *Onderstepoort J. Vet. Res.* 71: 99-105.

Mahan, S. M., D. Kumbula, M. J. Burridge & A. F. Barbet (1998). The inactivated *Cowdria ruminantium* vaccine for heartwater protects against heterologous strains and against laboratory and field tick challenge. *Vaccine* 16: 1203-1211.

Mahan, S. M., B. A. Allsopp, K. M. Kocan, G. H. Palmer & F. Jongejan (1999). Vaccine strategies for *Cowdria ruminantium* infections and their application to other ehrlichial infections. *Parasitol. Today* 15: 290-294.

Mahan, S. M., H. R. Andrew, N. Tebele, M. J. Burridge & A. F. Barbet (1995). Immunisation of sheep against heartwater with inactivated *Cowdria ruminantium*. *Res. Vet. Sci.* 58: 46-49.

Mahan, S. M., G. E. Smith, D. Kumbula, M. J. Burridge & A. F. Barbet (2001). Reduction in mortality from heartwater in cattle, sheep and goats exposed to field challenge using an inactivated vaccine. *Vet. Parasitol.* 97: 295-308.

Mahan, S. M., S. D. Waghela, T. C. McGuire, F. R. Rurangirwa, L. A. Wassink & A. F. Barbet (1992). A cloned DNA probe for *Cowdria ruminantium* hybridizes with eight heartwater strains and detects infected sheep. *J. Clin. Microbiol.* 30: 981-986.

Mahan, S. M., T. C. McGuire, S. M. Semu, M. V. Bowie, F. Jongejan, F. R. Rurangirwa & A. F. Barbet (1994). Molecular cloning of a gene encoding the immunogenic 21 kDa protein of *Cowdria ruminantium*. *Microbiology* 140: 2135-2142.

Mahan, S. M., N. Tebele, D. Mukwedeya, S. Semu, C. B. Nyathi, L. A. Wassink, P. J. Kelly, T. Peter & A. F. Barbet (1993). An immunoblotting diagnostic assay for heartwater based on the immunodominant 32-kilodalton protein of *Cowdria ruminantium* detects false positives in field sera. *J. Clin. Microbiol.* 31: 2729-2737.

Marcelino, I., M. F. Sousa, C. Verissimo, A. E. Cunha, M. J. Carrondo & P. M. Alves (2006). Process development for the mass production of *Ehrlichia ruminantium*. *Vaccine* 24: 1716-1725.

Marcelino, I., C. Verissimo, M. F. Sousa, M. J. Carrondo & P. M. Alves (2005). Characterization of *Ehrlichia ruminantium* replication and release kinetics in endothelial cell cultures. *Vet. Microbiol.* 110: 87-96.

Martinez, D., S. Coisne, C. Sheikboudou & F. Jongejan (1993). Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by ELISA. *Rev. Élev. Méd. vét. Pays trop.* 46: 115-120.

Martinez, D., J. Swinkels, E. Camus & F. Jongejan (1990). Comparaison de trois antigènes pour le sérodiagnostic de la cowdriose par immunofluorescence indirecte. *Rev. Élev. Méd. vét. Pays trop.* 43: 159-166.

Martinez, D., J. C. Maillard, S. Coisne, C. Sheikboudou & A. Bensaid (1994). Protection of goats against heartwater acquired by immunisation with inactivated elementary bodies of *Cowdria ruminantium*. *Vet. Immunol. Immunopathol.* 41: 153-163.

Martinez, D., J. M. Perez, C. Sheikboudou, A. Debus & A. Bensaid (1996). Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated *Cowdria ruminantium*. *Vet. Parasitol.* 67: 175-184.

Martinez, D., N. Vachiery, F. Stachurski, Y. Kandassamy, M. Raliniaina, R. Aprelon & A. Gueye (2004). Nested PCR for detection and genotyping of *Ehrlichia ruminantium*: use in genetic diversity analysis. *Ann. N. Y. Acad. Sci.* 1026: 106-113.

Mboloi, M. M., C. P. J. Bekker, C. Kruitwagen, M. Greiner & F. Jongejan (1999). Validation of the indirect MAP1-B enzyme-linked immunosorbent assay for diagnosis of experimental *Cowdria ruminantium* infection in small ruminants. *Clin. Diagn. Lab. Immunol.* 6: 66-72.

McBride, J. W., J. A. Comer & D. H. Walker (2003). Novel immunoreactive glycoprotein orthologs of *Ehrlichia* spp. *Ann. N. Y. Acad. Sci.* 990: 678-684.

McBride, J. W., X. Yu & D. H. Walker (2000a). A conserved, transcriptionally active *p28* multigene locus of *Ehrlichia canis*. *Gene* 254: 245-252.

McBride, J. W., X. J. Yu & D. H. Walker (2000b). Glycosylation of homologous immunodominant proteins of *Ehrlichia chaffeensis* and *Ehrlichia canis*. *Infect. Immun.* 68(1): 13-18.

Moshkovski, S. D. (1947). In "Comments by readers". *Science* 106: 62.

Mukhebi, A. W., T. Chamboko, C. J. O'Callagan, T. F. Peter, R. L. Kruska, G. F. Medley, S. M. Mahan & B. D. Perry (1999). An assessment of the economic impact of heartwater *(Cowdria ruminantium* infection) and its control in Zimbabwe. *Prev. Vet. Med.* 39: 173-189.

Mutunga, M., P. M. Preston & K. J. Sumption (1998). Nitric oxide is produced by *Cowdria ruminantium*-infected bovine pulmonary endothelial cells *in vitro* and is stimulated by gamma interferon. *Infect. Immun.* 66: 2115-2121.

Neitz, W. O. & R. Alexander (1945). Immunisation of cattle against heartwater and the control of the tick-borne diseases, red-water, gallsickness and heartwater. *J. S. Afr. Vet. Med. Assoc.* 12: 103-111.

Nyika, A., A. F. Barbet, M. J. Burridge & S. M. Mahan (2002). DNA vaccination with *map1* gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* 20: 1215-1225.

Nyika, A., S. M. Mahan, M. J. Burridge, T. C. McGuire, F. Rurangirwa & A. F. Barbet (1998). A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium*. *Parasite Immunol.* 20: 111-119.

Oberem, P. T. & J. D. Bezuidenhout (1987). Heartwater in hosts other than domestic ruminants. *Onderstepoort J. Vet. Res.* 54: 271-275.

Ohashi, N., Y. Rikihisa & A. Unver (2001). Analysis of transcriptionally active gene clusters of major outer membrane protein multigene family in *Ehrlichia canis* and *E. chaffeensis*. *Infect. Immun.* 69: 2083-2091.

Ohashi, N., A. Unver, N. Zhi & Y. Rikihisa (1998a). Cloning and characterization of multigenes encoding the immunodominant 30-kilodalton major outer membrane proteins of *Ehrlichia canis* and application of the recombinant protein for serodiagnosis. *J. Clin. Microbiol.* 36(9): 2671-2680.

Ohashi, N., N. Zhi, Y. Zhang & Y. Rikihisa (1998b). Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. *Infect. Immun.* 66: 132-139.

Pal U., L. Xin, T. Wang, R. Montgomery, N. Ramamoorthi, A. M. deSilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F. S. Kantor, S. Telford, J. F. Anderson & E. Fikrig (2004). TROSPA, an *Ixodes scapularis* Receptor for *Borrelia burgdorferi*. *Cell* 119:457-468.

Pal, U., R. R. Montgomery, D. Lusitani, P. Voet, V. Weynants, S. E. Malawista, Y. Lobet & E. Fikrig (2001). Inhibition of *Borrelia burgdorferi*-tick interactions *in vivo* by Outer Surface Protein A antibody. *J. Immunol.* 166: 7398-7403.

Palmer, G. H., G. Eid, A. F. Barbet, T. C. McGuire & T. F. McElwain (1994). The immunoprotective *Anaplasma marginale* Major Surface Protein 2 is encoded by a polymorphic multigene family. *Infect. Immun.* 62: 3808-3816.

Parkhill, J., M. Achtman, K. D. James, S. D. Bentley, C. Churcher, S. R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R.M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M. A. Quail, Rajandream M. A., K. M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B. G. Spratt & B. G. Barrell (2000). Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* 404: 451-2.

Peixoto, C. C., I. Marcelino, N. Vachiery, A. Bensaid, D. Martinez, M. J. Carrondo & P. M. Alves (2005). Quantification of *Ehrlichia ruminantium* by real time PCR. *Vet. Microbiol.* 107: 273-278.

Peter, T. F., S. L. Deem, A. F. Barbet, R. A. I. Norval, B. H. Simbi, P. J. Kelly & S. M. Mahan (1995). Development and evaluation of PCR assay for detection of low levels of *Cowdria ruminantium* infection in *Amblyomma* ticks not detected by DNA probe. *J.Clin.Microbiol.* 33: 166-172.

Pienaar, J. G. (1970). Electron microscopy of *Cowdria (Rickettsia) ruminantium* (Cowdry, 1926) in the endothelial cells of the vertebrate host. *Onderstepoort J. Vet. Res.* 37: 67-78.

Postigo, M., L. Bell-Sakyi, E. A. Paxton & K. J. Sumption (2002). Kinetics of experimental infection of sheep with *Ehrlichia ruminantium* cultivated in tick and mammalian cell lines. *Exp. Appl. Acar.* 28: 187-193.

Prozesky, L. & J. L. Du Plessis (1987). Heartwater. The development and life cycle of *Cowdria ruminantium* in the vertebrate host, ticks and cultured endothelial cells. *Onderstepoort J. Vet. Res.* 54: 193-6.

Prozesky, L., J. D. Bezuidenhout & C. L. Paterson (1986). Heartwater: an *in vitro* study of the ultrastructure of *Cowdria ruminantium*. *Onderstepoort J. Vet. Res.* 53: 153-159.

Purchase, H. (1945). A simple and rapid method for demonstrating *Rickettsia ruminantium* (Cowdry, 1925) in heartwater brains. *Vet. Rec.* 36: 413-414.

Ramamoorthi N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski & E. Fikrig (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host**.** *Nature* 436: 573-577.

Reddy, G. R., C. R. Sulsona, R. H. Harrison, S. M. Mahan, M. J. Burridge & A. F. Barbet (1996). Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from different geographical areas. *Clin. Diag. Lab. Immunol.* 3: 417-422.

Reddy, G. R., C. R. Sulsona, A. F. Barbet, S. M. Mahan, M. J. Burridge & A. R. Alleman (1998). Molecular characterization of a 28 kDa surface antigen gene family of the tribe Ehrlichiae. *Biochem. Biophys. Res. Comm.* 247: 636-643.

Rikihisa, Y. (2003). Mechanisms to create a safe haven by members of the family Anaplasmataceae. *Ann. N. Y. Acad. Sci.* 990: 548-55.

Roberts, D. M., M. Caimano, J. McDowell, M. Theisen, A. Holm, E. Orff, D. Nelson, S. Wikel, J. Radolf & R. T. Marconi (2002). Environmental regulation and differential production of members of the Bdr protein family of *Borrelia burgdorferi. Infect. Immun.* 70: 7033-7041.

Rossouw, M., A. W. Neitz, D. T. de Waal, J. L. Du Plessis, L. van Gas & S. Brett (1990). Identification of the antigenic proteins of *Cowdria ruminantium. Onderstepoort J. Vet. Res.* 57: 215-221.

Rurangirwa, F. R., D. Stiller, D. M. French & G. H. Palmer (1999). Restriction of major surface protein 2 (MSP2) variants during tick transmission of the ehrlichia *Anaplasma marginale*. *Proc. Natl. Acad. Sci. USA* 96: 3171-3176.

Schmidt, M. A., L. W. Riley & I. Benz (2003). Sweet new world: glycoproteins in bacterial pathogens. *Trends Microbiol.* 11: 554-561.

Schwan, T. G. & J. Piesman (2002). Vector interactions and molecular adaptations of Lyme disease and relapsing fever spirochetes associated with transmission by ticks. *Emerg. Infect. Dis.* 8: 115-121.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan & P.A. Rosa (1995). Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. U.S.A.* 92: 2909-2913.

Schwan T. G. & B. J. Hinnebusch (1998). Bloodstream- Versus Tick-Associated Variants of a Relapsing Fever Bacterium. *Science* 280:1938-40.

Shimada, M. K., M. H. Yamamura, P. M. Kawasaki, K. Tamekuni, M. Igarashi, O. Vidotto & M. C. Vidotto (2004). Detection of *Anaplasma marginale* DNA in larvae of *Boophilus microplus* ticks by polymerase chain reaction. *Ann. N. Y. Acad. Sci.* 1026: 95-102.

Semu, S. M., T. F. Peter, D. Mukwedeya, A. F. Barbet, F. Jongejan & S. M. Mahan (2001). Antibody responses to MAP 1B and other *Cowdria ruminantium* antigens are down regulated in cattle challenged with tick-transmitted heartwater. *Clin. Diagn. Lab. Immunol.* 8: 388-396.

Seville, M. (2001). A whole new way of looking at things: the use of Dark Reader technology to detect fluorophors. *Electrophoresis* 22: 814-828.

Shompole, S., F. R. Rurangirwa, A. Wambugu, J. Sitienel, D. M. Mwangi, A. J. Musoke, S. M. Mahan, C. W. Wells & T. C. McGuire (2000). Monoclonal antibody binding to a surface-exposed epitope on *Cowdria ruminantium* that is conserved among eight strains. *Clin. Diagn. Lab. Immunol.* 7: 983-986.

Sickmann, A., M. Mreyen & H. E. Meyer (2002). Identification of modified proteins by mass spectrometry. *IUBMB Life* 54: 51-57.

Simbi, B. H., T. F. Peter, M. J. Burridge & S. M. Mahan (2003). Comparing the detection of exposure to *Ehrlichia ruminantium* infection on a heartwater-endemic farm by the pCS20 polymerase chain reaction assay and an indirect MAP1-B enzyme linked immunosorbent assay. *Onderstepoort J. Vet. Res.* 70: 231-235.

Singu, V., H. Liu, C. Cheng & R. R. Ganta (2005). *Ehrlichia chaffeensis* expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. *Infect. Immun.* 73: 79-87.

Sirigireddy, K. & R. R. Ganta (2005). Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. *J. Mol. Diagn.* 7: 308-316.

Soldan, A. W., T. L. Norman, S. Masaka, E. A. Paxton, M. Edelsten & K. J. Sumption (1993). Seroconversion to *Cowdria ruminantium* of Malawi zebu calves, reared under different tick control strategies. *Rev. Elev. Med. vet. Pays Trop.* 46: 171-177.

Sulsona, C. R., S. M. Mahan & A. F. Barbet (1999). The *map1* gene of *Cowdria ruminantium* is a member of a multigene family containing both conserved and variable genes. *Biochem. Biophys. Res. Comm.* 257: 300-305.

Sumption, K. J., E. A. Paxton & L. Bell-Sakyi (2003). Development of a polyclonal competitive enzyme-linked immunosorbent assay for detection of antibodies to *Ehrlichia ruminantium*. *Clin. Diagn. Lab. Immunol.* 10: 910-916.

Taillardat-Bisch, A., D. Raoult & M. Drancourt (2003). RNA polymerase β-subunit-based phylogeny of *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp. and *Wolbachia pipientis*. *Int. J. Syst. Evol. Microbiol.* 53: 455-458.

Totté, P., D. Blankaert, P. Zilimwabagabo & J. Werenne (1993). Inhibition of *Cowdria ruminantium* infectious yield by interferons alpha and gamma in endothelial cells. *Rev. Elev. Med. vet. Pays Trop.* 46: 189-194.

Totté, P., D. J. McKeever, D. Martinez & A. Bensaid (1997). Analysis of T-cell responses in cattle immunized against heartwater by vaccination with killed elementary bodies of *Cowdria ruminantium*. *Infect. Immun.* 65: 236-241.

Totté, P., A. Bensaid, S. M. Mahan, D. Martinez & D. J. McKeever (1999). Immune responses to *Cowdria ruminantium* infections. *Parasitol. Today* 15: 286-290.

Uilenberg, G. (1983). Heartwater (*Cowdria ruminantium* infection): Current status. *Adv. Vet. Sci. Comp. Med.* 27: 428-455.

Uilenberg, G., E. Camus & N. Barré (1985). Quelques observations sur une souche de *Cowdria ruminantium* isolée en Guadaloupe (Antilles francaises). *Rev. Elev. Med. vet. Pays trop.* 38: 34-42.

Unver, A., Y. Rikihisa, R. W. Stich, N. Ohashi & S. Felek (2002). The *omp-1* major outer membrane multigene family of *Ehrlichia chaffeensis* is differentially expressed in canine and tick hosts. *Infect. Immun.* 70: 4701-4704.

Unver, A., N. Ohashi, T. Tajima, R. W. Stich, D. Grover & Y. Rikihisa (2001). Transcriptional analysis of *p30* Major Outer Membrane multigene family of *Ehrlichia canis* in dogs, ticks, and cell culture at different temperatures. *Infect. Immun.* 69: 6172-6178.

Vachiery, N., T. Lefrancois, I. Esteves, S. Molia, C. Sheikboudou, Y. Kandassamy & D. Martinez (2006). Optimisation of the inactivated vaccine dose against heartwater and *in vitro* quantification of *Ehrlichia ruminantium* challenge material. *Vaccine* 24: 4747-4756.

van Heerden, H., N. E. Collins, K. A. Brayton, C. Rademeyer & B. A. Allsopp (2004). Characterization of a major outer membrane protein multigene family in *Ehrlichia ruminantium*. *Gene* 330: 159-168.

van Kleef, M., A. W. H. Neitz & D. T. de Waal (1993). Isolation and characterization of antigenic proteins of *Cowdria ruminantium*. *Rev. Elev. Med. vet. Pays Trop.* 46: 157-164.

van Vliet, A. H. M., F. Jongejan, M. van Kleef & B. A. M. van der Zeijst (1994). Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. *Infect. Immun.* 62: 1451-1456.

van Vliet, A. H. M., B. A. M. van der Zeijst, E. Camus, S. M. Mahan, D. Martinez & F. Jongejan (1995). Use of a specific immunogenic region on the *Cowdria ruminantium* MAP1 protein in a serological assay. *J. Clin. Microbiol.* 33: 2405-2410.

Waghela, S. D., F. R. Rurangirwa, S. M. Mahan, C. E. Yunker, T. B. Crawford, A. F. Barbet, M. J. Burridge & T. C. McGuire (1991). A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. *J. Clin. Microbiol.* 29: 2571-2577.

Wang, X., Y. Rikihisa, T. H. Lai, Y. Kumagai, N. Zhi & S. M. Reed (2004). Rapid sequential changeover of expressed *p44* genes during the acute phase of *Anaplasma phagocytophilum* infection in horses. *Infect. Immun.* 72: 6852-6859.

Weiss, E. (1991). Biology of ehrlichiae. *Eur. J. Epidemiol.* 7: 253-8.

Winstanley, C. (2002). Spot the difference: applications of subtractive hybridisation to the study of bacterial pathogens. *J. Med. Microbiol.* 51: 459-467.

Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E.A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, A. S. Durkin, J. F. Kolonay, W.C. Nelson, Mohamoud J, P. Lee, C. Berry, M. B. Young, T. Utterback, J. Weidman, W. C. Nierman, I. T. Paulsen, K. E. Nelson, H. Tettelin, S. L. O'Neill & J. A. Eisen (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: A streamlined genome overrun by mobile genetic elements. *PloS Genet.* 2: E69.

Yang, M. & J. Sytkowski (1996). Cloning differentially expressed genes by linker capture subtraction. *Anal. Biochem.* 237: 109-114.

Yu, X. J., J. W. McBride & D. H. Walker (1999). Genetic diversity of the 28-kilodalton outer membrane protein gene in human isolates of *Ehrlichia chaffeensis. J. Clin. Microbiol.* 37: 1137-1143.

Yu, X., J. W. McBride, X. Zhang & D. H. Walker (2000). Characterization of the complete transcriptionally active *Ehrlichia chaffeensis* 28 kDa outer membrane protein multigene family. *Gene* 248: 59-68.

Yunker, C. E. (1996). Heartwater in sheep and goats: a review. *Onderstepoort J. Vet. Res.* 63: 159-170.

Yunker, C. E., S. M. Mahan, S. D. Waghela, T. C. McGuire, F. R. Rurangirwa, A. F. Barbet & L. A. Wassink (1993). Detection of *Cowdria ruminantium* by means of a DNA probe, pCS20 in infected bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in southern Africa. *Epidemiol. Infect.* 110: 95-104.

Zhang, J. Z., H. Guo, G. M. Winslow & X. J. Yu (2004a). Expression of members of the 28-kilodalton major outer membrane protein family of *Ehrlichia chaffeensis* during persistent infection. *Infect. Immun.* 72: 4336-4343.

Zhang, J., M. Sinha, B. Luxon & X. Yu (2004b). Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. *Infect. Immun.* 72: 498-507.

Zhi, N., N. Ohashi & Y. Rikihisa (1999). Multiple *p44* genes encoding Major Outer Membrane Proteins are expressed in the human granulocytic ehrlichiosis agent. *J. Biol. Chem.* 274: 17828-17836.

Zhi, N., N. Ohashi, T. Tajima, J. Mott, R. W. Stich, D. Grover, S. R. Telford III, Q. Lin & Y. Rikihisa (2002). Transcript heterogeneity of the *p44* multigene family in a human granulocytic ehrlichiosis agent transmitted by ticks. *Infect. Immun.* 70: 1175-1184.

Zweygarth, E., A. I. Josemans, F. van Strijp, L. Lopez-Rebollar, M. van Kleef & B. A. Allsopp (2005). An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge. *Vaccine* 23: 1695-1702.

Annexes

Annex 1: Temperature data from sheep in experimental infection 1 (Chapter 2, section 2.2.2.1)

Day	Sheep number /Temperature							Remarks
	147	**238**	**241**	**391**	**489**	**546**	**651**	
0	39,5	40,0	40,0	39,5	39,4	39,3	39,7	Each sheep was inoculated intravenously
1	39,4	40,0	39,6	39,3	39,5	39,4	39,7	with 1ml of 1:10 dilution of *E.ruminantium*
2	39,7	39,7	39,9	39,5	39,8	39,4	39,6	(CTVM Gardel) STAB1 on day 0
3	39,7	39,3	39,4	39,1	39,2	39,0	38,7	
4	39,4	39,2	39,2	39,3	39,8	39,4	39,6	
5	39,8	39,7	39,4	39,4	40,1	39,0	39,2	
6	40,0	39,9	39,7	39,9	39,8	39,2	39,6	
7	39,6	39,7	39,8	40,1	39,5	39,2	39,8	
8	39,7	39,7	39,7	39,6	39,6	39,1	39,3	
9	39,7	39,7	39,7	39,1	39,7	39,0	39,9	
10	39,5	38,9	39,7	39,4	39,3	39,3	39,5	
11	41,2	39,7	40,6	39,5	41,6	40,8	40,6	
12	40,7	41,0	40,3	40,7	41,2	41,1	40,8	
13	40,9	41,0	41,0	40,6	41,4	40,1	40,6	
14	42,0	41,3		41,2		41,5	41,1	
15	41,9			41,7		41,6	41,7	
16				41,0			41,0	

Annex 2: Post mortem findings from sheep in experimental infection 1 (Chapter 2, section 2.2.2.1).

Sheep	First day of fever	Max temp ℃ (day)	Day of euthanasia	Hydro-pericardium	Hydrothorax	Ascites	Brain smear
147	11	42.0 (14)	15	6ml	none	None	8%
238	12	41.3 (14)	14	15ml	trace	200ml	<0.1%
241	13	41.0 (13)	13*	15ml	20ml	some	<0.1%
391	14	41.7 (15)	16	7ml	trace	some	4%
489	11	41.6 (11)	13	5ml	trace	trace	<1%
546	11	41.6 (15)	15	9ml	trace	none	5%
651	12	41.7 (15)	16	14ml	trace	none	5%

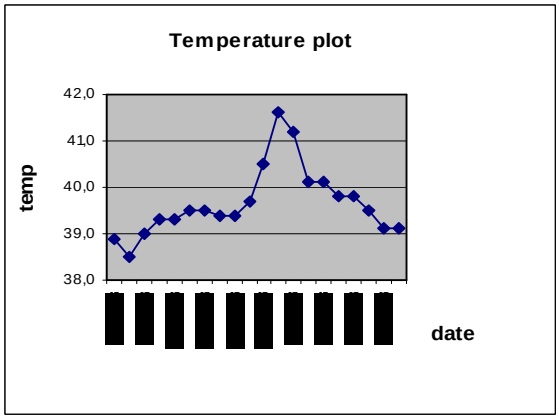
*euthanased on clinical (respiratory) signs

Annex 3: Acquisition feeding 1, temperatures and ELISA results from sheep **3148** (Chapter 4, sections:4.2.3.1 and 4.3.6.1)

3.1 Acquisition feeding

Sheep # 3148				
date	temp	blood	serum	remarks
28.11.03	38,9	1	1	Inoculated intravenously with 1ml of 1:10 dilution of *E. ruminantium* (CTVM Gardel) STAB1
29.11.03	38,5			
30.11.03	39,0			
01.12.03	39,3			
02.12.03	39,3	1	1	
03.12.03	39,5			Nymphs applied (EL-26/2 KO3)
04.12.03	39,5			Nymphs applied (EL-22/23-KO3)
05.12.03	39,4			Nymphs applied (EL-12/13-KO3)
06.12.03	39,4			
07.12.03	39,7			
08.12.03	40,5			
09.12.03	41,6	1	1	engorged nymphs collected
10.12.03	41,2	1		engorged nymphs collected*/5 ml Engemycine
11.12.03	40,1	1		engorged nymphs collected after treat./5 ml Engemycine
12.12.03	40,1			engorged nymphs collected after treat./5 ml Engemycine
13.12.03	39,8			5 ml Engemycine
14.12.03	39,8			5 ml Engemycine
15.12.03	39,5			Final removing of ticks
16.12.03	39,1	1	1	
17.12.03	39,1			* Total of adults recovered after moulting:
				37 females
23.12.03		1	1	12 males
30.12.03		1	1	
06.01.04		1	1	
13.01.04		1	1	

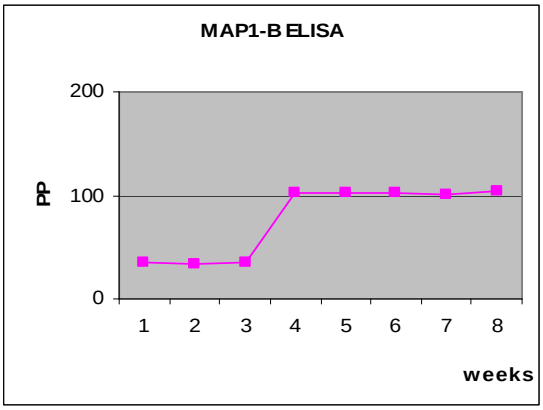
3.2 Temperature data (sheep 3148)



Sheep # 3148	
date	temp
28.11.03	38,9
29.11.03	38,5
30.11.03	39,0
01.12.03	39,3
02.12.03	39,3
03.12.03	39,5
04.12.03	39,5
05.12.03	39,4
06.12.03	39,4
07.12.03	39,7
08.12.03	40,5
09.12.03	41,6
10.12.03	41,2
11.12.03	40,1
12.12.03	40,1
13.12.03	39,8
14.12.03	39,8
15.12.03	39,5
16.12.03	39,1
17.12.03	39,1

3.3 ELISA results (sheep 3148)

	MAP1-B Elisa		
Date	samples	mean OD	PP
	pos	0,6144	100
	neg	0,2166	35,25
28.11.03	1	0,2215	36,05
02.12.03	2	0,2100	34,17
09.12.03	3	0,2210	35,97
16.12.03	6	0,6340	103,19
23.12.03	7	0,6325	102,94
30.12.03	8	0,6260	101,88
06.01.04	9	0,6240	101,56
13.01.04	10	0,6380	103,84

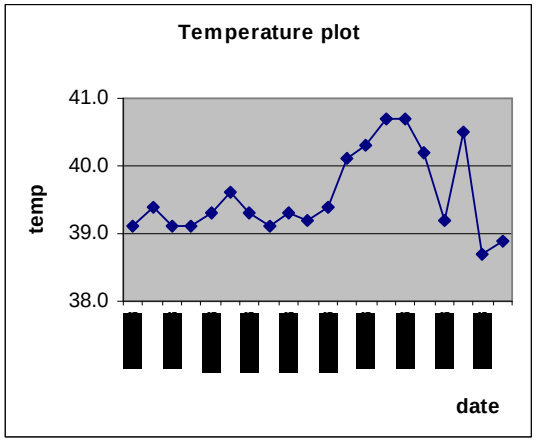


Annex 4: Acquisition feeding 1, temperatures and ELISA results from sheep **3175** (Chapter 4, sections:4.2.3.1 and 4.3.6.1)

4.1 Acquisition feeding

Sheep # 3175				
date	temp	blood	serum	Remarks
28.11.03	39.1	1	1	Inoculated intravenously with 1ml of 1:10 dilution of *E. ruminantium* (CTVM Gardel) STAB1
29.11.03	39.4			
30.11.03	39.1			
01.12.03	39.1			
02.12.03	39.3	1	1	
03.12.03	39.6			Nymphs applied (EL-28/29-KO3)
04.12.03	39.3			Nymphs applied (EL-16/17/18/19-KO9)
05.12.03	39.1			Nymphs applied (EL-10/11-KO3)
06.12.03	39.3			
07.12.03	39.2			
08.12.03	39.4			
09.12.03	40.1	1	1	engorged nymphs collected
10.12.03	40.3	1		engorged nymphs collected
11.12.03	40.7	1		engorged nymphs collected*/5 ml Engemycine
12.12.03	40.7			engorged nymphs collected after treat./5 ml Engemycine
13.12.03	40.2			5 ml Engemycine
14.12.03	39.2			5 ml Engemycine
15.12.03	40.5			Final removing of ticks
16.12.03	38.7	1	1	
17.12.03	38.9			
				* Total of adults recovered after moulting:
23.12.03		1	1	48 females
30.12.03		1	1	13 males
06.01.04		1	1	
13.01.04		1	1	

4.2 Temperature data (sheep 3175)

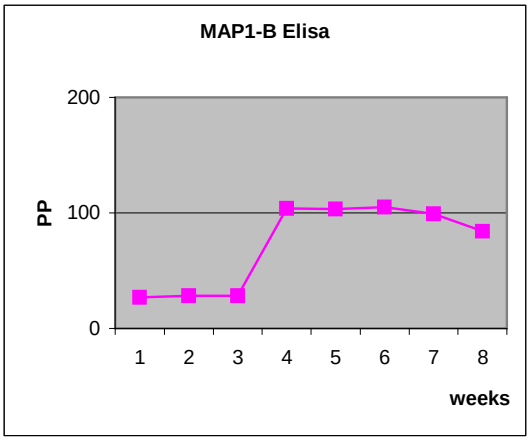


date	temp
28.11.03	39.1
29.11.03	39.4
30.11.03	39.1
01.12.03	39.1
02.12.03	39.3
03.12.03	39.6
04.12.03	39.3
05.12.03	39.1
06.12.03	39.3
07.12.03	39.2
08.12.03	39.4
09.12.03	40.1
10.12.03	40.3
11.12.03	40.7
12.12.03	40.7
13.12.03	40.2
14.12.03	39.2
15.12.03	40.5
16.12.03	38.7
17.12.03	38.9

4.2 ELISA results (sheep 3175)

MAP1-B Elisa

date	samples	mean OD	PP
	pos	0.6144	100
	neg	0.2166	35.25
28.11.03	1	0.1655	26.93
02.12.03	2	0.1730	28.15
09.12.03	3	0.1740	28.32
16.12.03	6	0.6390	104.00
23.12.03	7	0.6360	103.51
30.12.03	8	0.6450	104.98
06.01.04	9	0.6100	99.28
13.01.04	10	0.5170	84.14



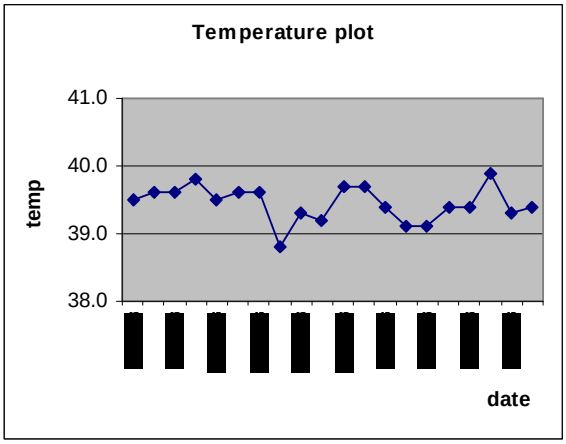
Annex 5: Acquisition feeding 1, temperatures and ELISA results from sheep **3183** (Chapter 4, section:4.3.6.1)

5.1 Acquisition feeding

Sheep # 3183

date	temp	blood	serum	remarks
28.11.03	39.5	1	1	
29.11.03	39.6			
30.11.03	39.6			
01.12.03	39.8			
02.12.03	39.5	1	1	
03.12.03	39.6			Nymphs applied (EL-24/25-KO3)
04.12.03	39.6			Nymphs applied (EL-20/21-KO9)
05.12.03	38.8			Nymphs applied (EL-14/15-KO3)
06.12.03	39.3			
07.12.03	39.2			
08.12.03	39.7			
09.12.03	39.7	1	1	engorged nymphs collected
10.12.03	39.4	1		engorged nymphs collected
11.12.03	39.1	1		engorged nymphs collected
12.12.03	39.1			engorged nymphs collected
13.12.03	39.4			
14.12.03	39.4			
15.12.03	39.9			Final removing of ticks*
16.12.03	39.3	1	1	
17.12.03	39.4			
				* Total of adults recovered after moulting:
23.12.03		1	1	43 females
30.12.03		1	1	5 males
06.01.04		1	1	
13.01.04		1	1	

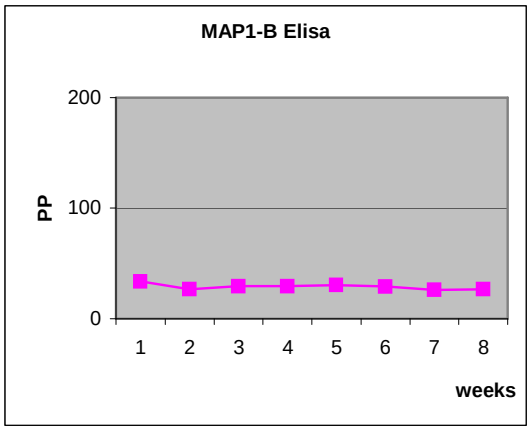
5.1 Temperature data (sheep 3183)



date	temp
28.11.03	39.5
29.11.03	39.6
30.11.03	39.6
01.12.03	39.8
02.12.03	39.5
03.12.03	39.6
04.12.03	39.6
05.12.03	38.8
06.12.03	39.3
07.12.03	39.2
08.12.03	39.7
09.12.03	39.7
10.12.03	39.4
11.12.03	39.1
12.12.03	39.1
13.12.03	39.4
14.12.03	39.4
15.12.03	39.9
16.12.03	39.3
17.12.03	39.4

5.3 ELISA results (sheep 3183)

	MAP1-B Elisa		
date	samples	mean OD	PP
	pos	0.6144	100
	neg	0.2166	35.25
28.11.03	1	1	33.61
02.12.03	2	2	26.69
09.12.03	3	3	29.45
16.12.03	6	6	29.37
23.12.03	7	7	30.35
30.12.03	8	8	29.05
06.01.04	9	9	25.96
13.01.04	10	10	26.61



Annex 6: Transmission feeding 1, temperatures and ELISA results from sheep **3149** (Chapter 4, section:4.3.6.2)

Sheep # 3149				
date	temp	blood	serum	remarks
02/04/2004	39.3	1	1	12 colony uninfected males were placed in bag1
03/04/2004	39.7	1		
04/04/2004	39.4	1		
05/04/2004	39.6	1		
06/04/2004	39.2	1		
07/04/2004	39.4	1		
08/04/2004	39.3	1		
09/04/2004	39.8	1	1	
10/04/2004	39.2	1		
11/04/2004	39.3	1		30 infected females and 10 infected males
12/04/2004	39.8	1		Detaching ticks
13/04/2004	39.8	1		Detaching ticks
14/04/2004	39.5	1		Detaching ticks
15/04/2004	39.8	1		Detaching ticks
16/04/2004	39.4	1	1	Detaching ticks
17/04/2004	40.1	1		
18/04/2004	39.8	1		
19/04/2004	39.2	1		
20/04/2004	39.2	1		
21/04/2004	39.6	1		
22/04/2004	39.3	1		
23/04/2004	39.5	1	1	
24/04/2004	39.7	1		
25/04/2004	39.5	1		
26/04/2004	39.2	1		
27/04/2004	39.6	1		
28/04/2004	38.8	1		
29/04/2004	39.5	1		
30/04/2004	39.5	1	1	
01/05/2004	39.0	1		
02/05/2004	39.0	1		
03/05/2004	39.6	1		
04/05/2004	39.2	1		
05/05/2004	39.6	1		
06/05/2004	38.9	1		
07/05/2004	39.3	1	1	
08/05/2004	39.5	1		
09/05/2004	39.4	1		
10/05/2004	39.2	1		
11/05/2004	38.9	1		
12/05/2004	39.2	1		
13/05/2004	39.6	1		
14/05/2004	40.3	1	1	
15/05/2004	39.4	1	1	
21/05/2004		1	1	Sheep euthanased, ELISA Neg.

Annex 7: Transmission feeding 1, temperatures and ELISA results from sheep **3190** (Chapter 4, section: 4.3.6.2)

Sheep # 3190				
date	temp	blood	serum	remarks
09/04/2004	39.2	1	1	12 colony uninfected males were placed in bag 1
10/04/2004	39.2	1		
11/04/2004	39.4	1		
12/04/2004	39.5	1		
13/04/2004	39.5	1		
14/04/2004	39.3	1		
15/04/2004	39.4	1		
16/04/2004	39.3	1	1	
17/04/2004	39.7	1		
18/04/2004	39.5	1		30 infected females and 10 infected males
19/04/2004	39.3	1		Detaching ticks
20/04/2004	39.5	1		Detaching ticks
21/04/2004	39.5	1		Detaching ticks
22/04/2004	39.2	1		Detaching ticks
23/04/2004	39.3	1	1	Detaching ticks
24/04/2004	39.4	1		
25/04/2004	39.4	1		
26/04/2004	39.1	1		
27/04/2004	39.3	1		
28/04/2004	39.5	1		
29/04/2004	39.2	1		
30/04/2004	39.4	1	1	
01/05/2004	39.5	1		
02/05/2004	39.4	1		
03/05/2004	39.6	1		
04/05/2004	39.5	1		
05/05/2004	39.4	1		
06/05/2004	39.5	1		
07/05/2004	39.5	1	1	
08/05/2004	39.3	1		
09/05/2004	39.3	1		
10/05/2004	39.5	1		
11/05/2004	39.1	1		
12/05/2004	39.1	1		
13/05/2004	39.4	1		
14/05/2004	39.6	1	1	
21/05/2004	39.1	1	1	Sheep euthanased, ELISA Neg.

Annex 8: Transmission feeding 1 and temperature data from sheep **3180** (Chapter 4, section: 4.3.6.2)

Sheep # 3180

date	temp	blood	serum	remarks
09/03/2004	39.8	1	1	Adult males were applied
10/03/2004	39.7	1		
11/03/2004	40.0	1		
12/03/2004	39.9	1		All males attached and feeding
13/03/2004	39.9	1		
14/03/2004	39.9	1		30 females were placed
15/03/2004	39.9	1		Females detached
16/03/2004	39.4	1	1	Females detached
17/03/2004	39.6	1		Females detached
18/03/2004	39.9	1		Females detached
19/03/2004	39.9	1		Females detached
20/03/2004	39.5	1		
21/03/2004	39.2	1		
22/03/2004	39.0			
23/03/2004		1	1	
30/03/2004		1	1	

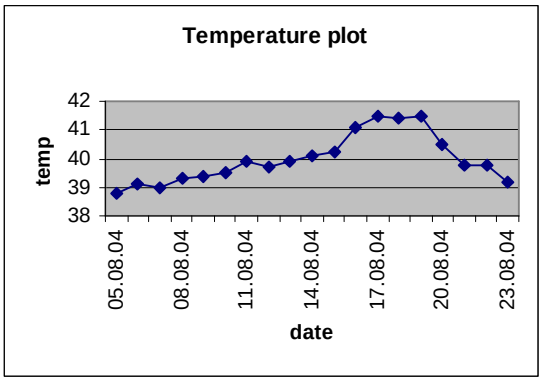
Annex 9: Acquisition feeding 2, temperatures and ELISA results from sheep **3154** (Chapter 2, section: 2.2.2.2; Chapter 4, section: 4.3.7.1)

9.1 Acquisition feeding 2 (sheep 3154)

Sheep # 3154

date	temp	blood	serum	remarks
05.08.04	38.8	1	1	Inoculated intravenously with 1ml of 1:10 dilution of *E. ruminantium* (CTVM Gardel) STAB1
06.08.04	39.1	1		
07.08.04	39.0	1		
08.08.04	39.3	1		
09.08.04	39.4	1		
10.08.04	39.5	1		Larvae applied (JL 04-Left ear)
11.08.04	39.9	1		Larvae applied (JL 05/06-Left and right ear)
12.08.04	39.7	1	1	Larvae applied (JL 07-Right ear)
13.08.04	39.9	1		
14.08.04	40.1	1		
15.08.04	40.2	1		
16.08.04	40.9/41.3	1		First day of fever
17.08.04	41.9/41.2	1		Engorged larvae collected (1 tube=50 ticks)
18.08.04	41.2/41.6	1		Engorged larvae collected (6T morning / 6T evening)
19.08.04	41.5/41.5	1	1	Engorged larvae collected (3T). 7 ml of engemycine
20.08.04	40.7/40.4	1		7 ml Engemycine. Final collection of ticks
21.08.04	39.7/39.8	1		7 ml Engemycine
22.08.04	39.8	1		
23.08.04	39.2	1		
26.08.04		1	1	
02.09.04		1	1	
09.09.04		1	1	
16.09.04		1	1	
23.09.04			1	
				* Total of nymphs recovered after moulting:
				Approx: 800 nymphs

9.2 Temperature data (sheep 3154)

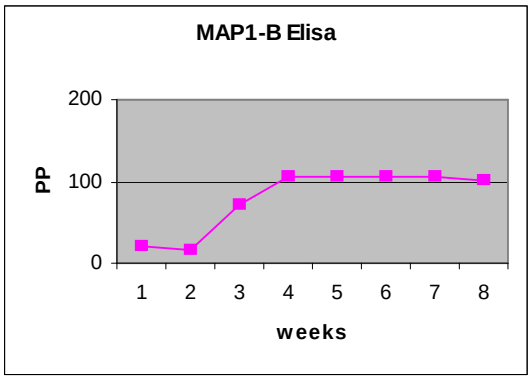


date	temp
05.08.04	38.8
06.08.04	39.1
07.08.04	39.0
08.08.04	39.3
09.08.04	39.4
10.08.04	39.5
11.08.04	39.9
12.08.04	39.7
13.08.04	39.9
14.08.04	40.1
15.08.04	40.2
16.08.04	40.9/41.3
17.08.04	41.9/41.2
18.08.04	41.2/41.6
19.08.04	41.5/41.5
20.08.04	40.7/40.4
21.08.04	39.7/39.8
22.08.04	39.8
23.08.04	39.2

9.3 ELISA results (sheep 3154)

MAP1-B Elisa

date	samples	mean OD	PP
	pos	1.8880	100
	neg	0.4110	21.76
05.08.04	1	0.3920	20.76
12.08.04	8	0.3060	16.20
19.08.04	15	1.3350	70.7
26.08.04	22	1.9960	105.72
02.09.04	23	2.0060	106.25
09.09.04	24	1.9760	104.66
16.09.04	25	1.981	104.9
23.09.04	26	1.891	100.15



Annex 10: Transmission feeding 2, temperatures and ELISA results from sheep **3471** (Chapter 4, section: 4.3.7.2)

Sheep # 3471				
date	temp	blood	serum	remarks
10.10.04	39.2	1	1	Tick application (Nymphs)
11.10.04	39.2	1		60 ticks were detached
12.10.04	38.6	1		
13.10.04	38.8	1		60 ticks were detached
14.10.04	38.7	1	1	
15.10.04	38.6	1		Ticks were collected full engorged
16.10.04	38.6	1		Engorged ticks collected
17.10.04	39.1	1		Engorged ticks collected
18.10.04	38.7	1		Engorged ticks collected
19.10.04	38.7	1		Engorged ticks collected
20.10.04	39.0	1		
21.10.04	38.9	1	1	
22.10.04	39.2	1		
23.10.04	38.6	1		
24.10.04	38.9	1		
25.10.04	39.2	1		
26.10.04	38.9	1		
27.10.04	38.9	1		
28.10.04	39.2	1	1	
29.10.04	38.5	1		
30.10.04	38.9	1		
31.10.04	38.9	1		
01.11.04	38.8	1		
02.11.04	38.9	1		
03.11.04	39.1	1		
04.11.04	38.9	1	1	
05.11.04	39.0	1		
06.11.04	39.0	1		
07.11.04	39.1	1		
08.11.04	39.3	1		
09.11.04	39.1	1		
10.11.04	39.3	1		
11.11.04	39.3	1	1	
12.11.04	39.1	1		
13.11.04	39.1	1		
14.11.04	39.1	1		
15.11.04	39.0	1		
17.11.04	39.2	1		ELISA neg.

10.1 (*continued…*)

Sheep #3471				
date	temp.	blood	serum	remarks
19.11.04	38.9	1		
22.11.04	39	1		
24.11.04	39.1	1	1	Sheep was inoculated with 2 vials of *E. ruminantium* (Gardel isolate) infected blood (stabilate CR366)
25.11.04	39.4	1	1	
26.11.04	39.9	1		
27.11.04	39.1	1		
28.11.04	38.9	1		
29.11.04	38.9	1		
30.11.04	39.1	1		
01.12.04	39.1	1		
02.12.04	38.6	1	1	
03.12.04	39.0	1		
04.12.04	38.6	1		
05.12.04	40.1	1		
06.12.04	40.3/40.5	1		
07.12.04	40.8/41.0	1		
08.12.04	41.7/41.7	1		
09.12.04	42.1	1	1	
10.12.04	41.2	1		
11.12.04	40.4	1		Sheep showed increased breathing
12.12.04	40.0	1		Sheep showed nervous symptoms and died
				Ehrlichia was found in brain smears and PCR pos.

Annex 11: Acquisition feeding 3 and temperature data from sheep **3464**

(Chapter 4, section:4.3.8.1)

Sheep # 3464

date	temp	blood	serum	remarks
19.01.05	39.6	1	1	Sheep was inoculated with 2 vials of *E. ruminantium* (Gardel isolate) infected blood (stabilate CR366)
20.01.05	39.5	1		
21.01.05	39.2	1		
22.01.05	38.5	1		
23.01.05	39.1	1		
24.01.05	38.6	1		
25.01.05	38.6	1		Nymphs applied (Bag 1)
26.01.05	38.6	1	1	Nymphs applied (Bag 2)
27.01.05	38.6	1		Nymphs applied (Bag 1)
28.01.05	38.7	1		Nymphs applied (Bag2)
29.01.05	38.6			
30.01.05	40.0/40.1			6 engorged ticks collected
31.01.05	39.8/39.7	1		Engorged ticks collected (35 bag1 + 27 bag2)
01.02.05	**39.8/40.0**	**1**		**Engorged ticks collected (26 bag1 + 40 bag2)** first day of fever
02.02.05	**41.0/41.8**	**1**	**1**	**Engorged ticks collected (17 bag1 + 29 bag2)**
03.02.05	**41.9**			**Engorged ticks collected (6).** 1st day of treatment
04.02.05	39.5			2nd day of treatment
05.02.05	ND			Sheep died (Brain smears were taken on Mon. and showed E.r.-like bacteria; PCR from brain was clearly positive for E.r.)
				Total of recovered adults:
				From batch 01/02/05: 64 adults and 2 dead: 35M/29F
				From batch 02/02/05: 45 adults and 1 dead: 16M/29F
				From batch 03/02/05: 6 adults: 2M/4F

Annex 12: Transmission feeding 3 and temperature data from sheep **3456**

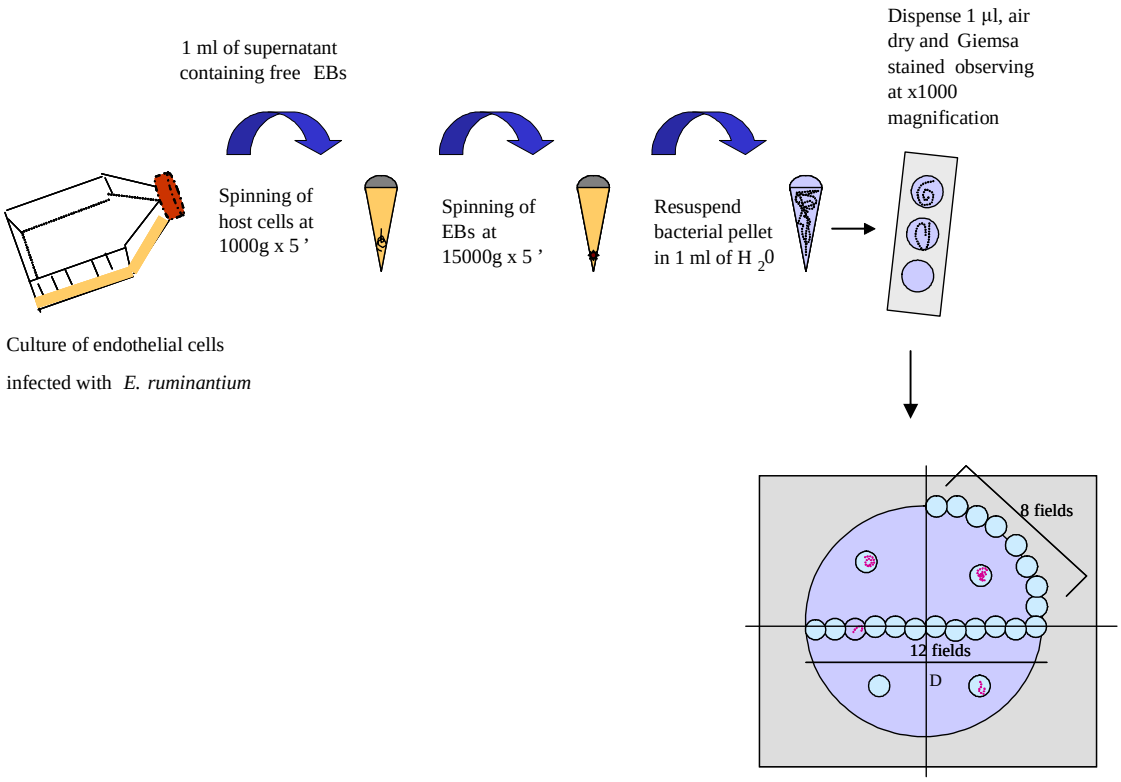
(Chapter 2, section:2.2.2.2; Chapter 4, section:4.3.8.2)

Sheep # 3456

date	temp	blood	serum	remarks
04.04.05	38.6	1	1	15 uninfected males (EHL6) applied in Bag1
05.04.05	38.9			
06.04.05	38.6			
07.04.05	38.6			
08.04.05	38.0			
09.04.05	38.4			
10.04.05	38.4	1	1	Applied: 28 infected females (2.2.05/3.2.05) in Bag1;
11.04.05*	39.4	1		18 infected males (2.2.05/3.2.05) in Bag2 and
12.04.05*	38.8	1		19 infected males (1.02.05) in Bag 3
13.04.05*	39.2	1		
14.04.05*	38.6	1	1	20 infected females (1.2.05) were placed in Bag3
15.04.05*	38.8	1		
16.04.05	39.4	1		
17.04.05	38.5	1		
18.04.05	38.4	1		
19.04.05	38.6	1		
20.04.05	38.8	1		
21.04.05	38.7	1	1	
22.04.05	39.4	1		
23.04.05	40.6/41.2	1		
24.04.05	ND			Sheep was found dead. All ticks left on sheep were removed and store in ethanol

* Ticks were detached for analysis

Annex 13: Quantification of *E. ruminantium* in Giemsa-stained preparations by light microscopy (Chapter 2, section 2.2.6.1)



Annex 13: Calculation of nº of EBs in 1 µl of suspension was done by counting the number of bacteria in a determined number of fields (i.e. 3 fields in the edge of the circle, where bacteria were more abundant, and i.e. 10 internal fields), in triplicate, and then extrapolating the number of bacteria based on the total number of fields in the circle.

The total number of fields in the circle was calculated as follows:

Total N^o fields=N^o of fields in the internal area circle + N^o of fields round the edge

[Diameter of the circle (D)= 1,5 cm (measured with a ruler)]

r=D/2

N^o of fields in the edge=32

N^o of fields in the internal area=Area of internal area (Ai)/Area of a small circle (Asc)

Ai=Total area circle (TA)-Area edge (AE)

$TA=\pi \times r^2 = 3,14 \times (0,75)^2 = 1,8\ cm^2$

AE= Area small circle (Asc) x 32 fields/2

$Asc=\pi \times rsc^2 = 3,14 \times (0.063)^2 = 0,01\ cm^2$
[Diameter small circle= D/12 fields= 1,5 cm/12= 0,125 cm]

$AE=0,01\ cm^2 \times 32/2= 0,2\ cm^2$

$Ai= 1,8\ cm^2 - 0,2\ cm^2 = 1,6\ cm^2$

No of fields in the internal area= $1,6\ cm^2/0,01\ cm^2$= 160 fields in the internal area

Total n^o fields=160 + 32= 192

i.e.

	# of bacteria		
Prep # 1/edge counting in three fields	1	2	3
1	432	389	438
2	270	328	349
3	233	600	301

Average=3339 total counts/ 9 fields=371 bacteria/field

# of bacteria

Average=450 total counts/ 30 fields=15 bacteria/field

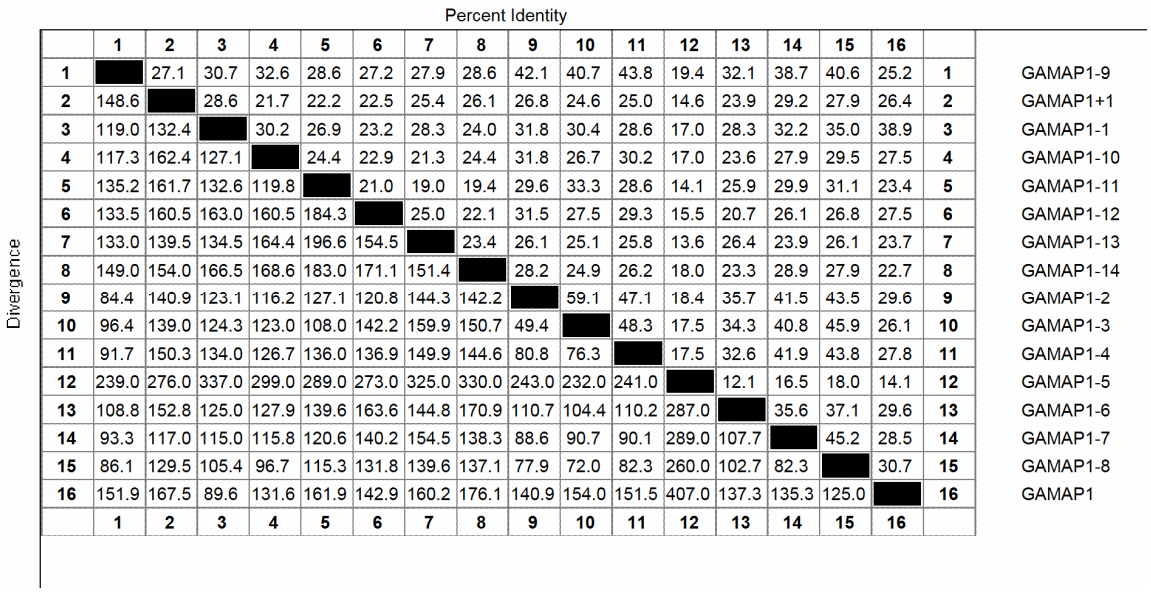
Prep#1/internal counting in 5 fields	1	2	3	Prep#1/internal counting in 5 fields	1	2	3
1	1	9	14	6	5	1	1
2	11	38	18	7	1	33	34
3	15	19	30	8	3	9	14
4	5	19	22	9	10	5	1
5	1	8	1	10	13	60	24

371 bacteria ----------1 fields edge area
x----------32 fields edge area
x= 11872 bacteria in the edge

15 bacteria----------1 field internal area
x----------192 fields internal area
x=2880 bacteria in the internal area

Total # of bacteria in 1µl of suspension= 11872 + 2880= 14752

Annex 14: Protein sequence distances within the MAP1 cluster (Chapter 5)



Percent Identity

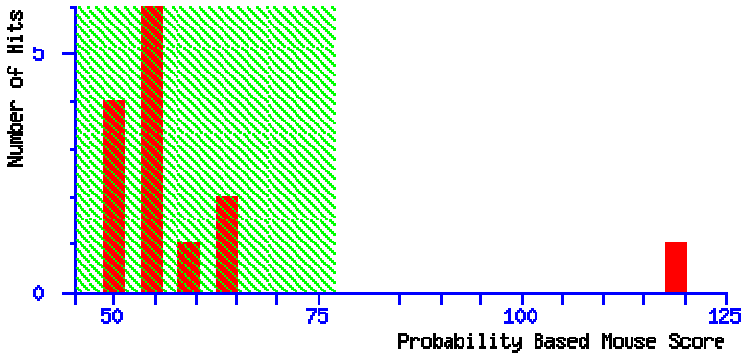
Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1		27.1	30.7	32.6	28.6	27.2	27.9	28.6	42.1	40.7	43.8	19.4	32.1	38.7	40.6	25.2	**1**	GAMAP1-9
2	148.6		28.6	21.7	22.2	22.5	25.4	26.1	26.8	24.6	25.0	14.6	23.9	29.2	27.9	26.4	**2**	GAMAP1+1
3	119.0	132.4		30.2	26.9	23.2	28.3	24.0	31.8	30.4	28.6	17.0	28.3	32.2	35.0	38.9	**3**	GAMAP1-1
4	117.3	162.4	127.1		24.4	22.9	21.3	24.4	31.8	26.7	30.2	17.0	23.6	27.9	29.5	27.5	**4**	GAMAP1-10
5	135.2	161.7	132.6	119.8		21.0	19.0	19.4	29.6	33.3	28.6	14.1	25.9	29.9	31.1	23.4	**5**	GAMAP1-11
6	133.5	160.5	163.0	160.5	184.3		25.0	22.1	31.5	27.5	29.3	15.5	20.7	26.1	26.8	27.5	**6**	GAMAP1-12
7	133.0	139.5	134.5	164.4	196.6	154.5		23.4	26.1	25.1	25.8	13.6	26.4	23.9	26.1	23.7	**7**	GAMAP1-13
8	149.0	154.0	166.5	168.6	183.0	171.1	151.4		28.2	24.9	26.2	18.0	23.3	28.9	27.9	22.7	**8**	GAMAP1-14
9	84.4	140.9	123.1	116.2	127.1	120.8	144.3	142.2		59.1	47.1	18.4	35.7	41.5	43.5	29.6	**9**	GAMAP1-2
10	96.4	139.0	124.3	123.0	108.0	142.2	159.9	150.7	49.4		48.3	17.5	34.3	40.8	45.9	26.1	**10**	GAMAP1-3
11	91.7	150.3	134.0	126.7	136.0	136.9	149.9	144.6	80.8	76.3		17.5	32.6	41.9	43.8	27.8	**11**	GAMAP1-4
12	239.0	276.0	337.0	299.0	289.0	273.0	325.0	330.0	243.0	232.0	241.0		12.1	16.5	18.0	14.1	**12**	GAMAP1-5
13	108.8	152.8	125.0	127.9	139.6	163.6	144.8	170.9	110.7	104.4	110.2	287.0		35.6	37.1	29.6	**13**	GAMAP1-6
14	93.3	117.0	115.0	115.8	120.6	140.2	154.5	138.3	88.6	90.7	90.1	289.0	107.7		45.2	28.5	**14**	GAMAP1-7
15	86.1	129.5	105.4	96.7	115.3	131.8	139.6	137.1	77.9	72.0	82.3	260.0	102.7	82.3		30.7	**15**	GAMAP1-8
16	151.9	167.5	89.6	131.6	161.9	142.9	160.2	176.1	140.9	154.0	151.5	407.0	137.3	135.3	125.0		**16**	GAMAP1
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**		

Annex 14: Percentages were calculated using DNAstar (MegAlign/ClustalV) based on *E. ruminantium* (Gardel) sequences.

Annex 15: Mascot search results from BUE spot 1 (Chapter 5)

Probability Based Mowse Score: Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 77 are significant (p<0.05).



Score: 117; MAP1 CAI28368 from Ehrlichia ruminantium str. Gardel

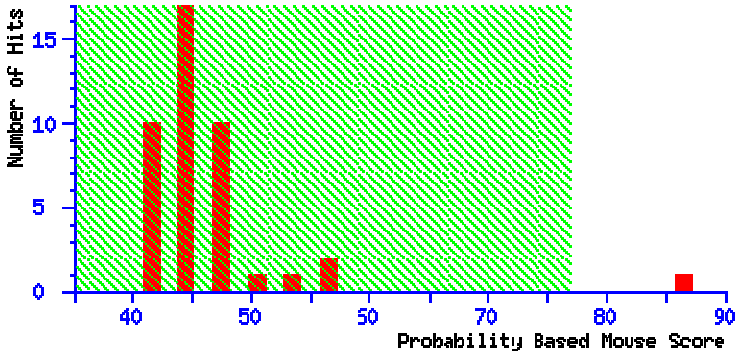
Nominal mass (M_r): **33410**; Calculated pI value: **5.74**
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **20**
Number of mass values matched: **9**
Sequence Coverage: **25%**

Matched peptides shown in **Bold Red**

1 MLLFFTSTIV NLFVIIRCNM NCKKIFITST LISLVSFLPG VSFSDVIQED
51 SSPAGSVYIS AK**YMPTASHF GK**MSIKEDSK **NTQTVFGLK**K **DWDGVKTPSS**
101 **DSGNNSIIFT EKDYSFK**YEN NPFLGFAGAI GYSMNGPR**IE FEVSYETFDV**
151 **KNPGGNYK**ND AHMYCALDTG TPGSTQGATL NSSVMVKNEN LTDIALMLNA
201 CYDITLEGMP VSPYVCAGIG TDLVSVINAT NPK**LSYQGK**L GISYSINPEA
251 SIFIGGHFHR **VIGNEFK**DIT TSKIFTSTGK LATAASPGFA SATLDVCHFG
301 IEIGGRFVF

No match to: 656.0653, 672.0779, 842.5358, 1094.2680, 1154.5730, 1160.5710, 1396.7320, 1695.8570, 1785.9040, 1865.9860, 1869.9520

Annex 16. Mascot search results from BUE spot 2 (Chapter 5)



Score: **85;** **MAP1** CAI28368 from Ehrlichia ruminantium str. Gardel

Nominal mass (M_r): **33752**; Calculated pI value: **5.74**

Fixed modifications: Carbamidomethyl (C)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **18**

Number of mass values matched: **6**

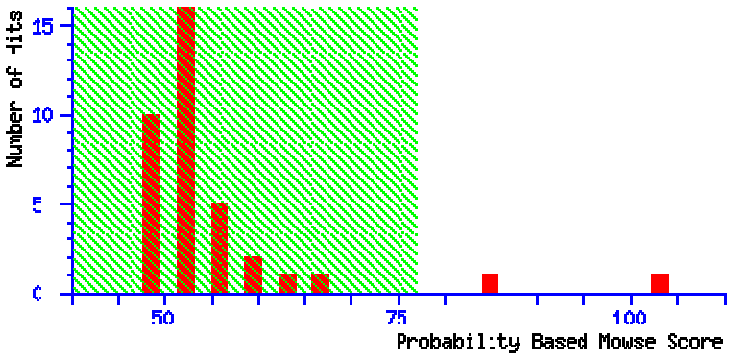
Sequence Coverage: **33%**

Matched peptides shown in **Bold Red**

1 MLLFFTSTIV NLFVIIRCNM NCKKIFITST LISLVSFLPG VSFSDVIQED
51 SSPAGSVYIS AK**YMPTASHF GK**MSIKEDSK **NTQTVFGLK**K DWDGVK**TPSS**
101 **DSGNNSIIFT EK**DYSFKYEN NPFLGFAGAI GYSMNGPR**IE FEVSYETFDV**
151 **K**NPGGNYK**ND AHMYCALDTG TPGSTQGATL NSSVMVK**NEN LTDIALMLNA
201 CYDITLEGMP VSPYVCAGIG TDLVSVINAT NPKLSYQGKL GISYSINPEA
251 SIFIGGHFHR VIGNEFKDIT TSKIFTSTGK **LATAASPGFA SATLDVCHFG**
301 **IEIGGR**FVF

No match to: 650.0337, 666.0177, 842.4888, 861.0818, 1154.5050, 1781.8930, 1865.8760, 2211.1070, 2222.1190, 2225.1290, 2239.1560, 2291.9880

Annex 17. Mascot search results from BUE spot 3 (Chapter 5)



Score: 101; MAP1 CAI28368 from Ehrlichia ruminantium str. Gardel

Nominal mass (M_r): **33752**; Calculated pI value: **5.74**

Fixed modifications: Carbamidomethyl (C)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **16**

Number of mass values matched: **7**

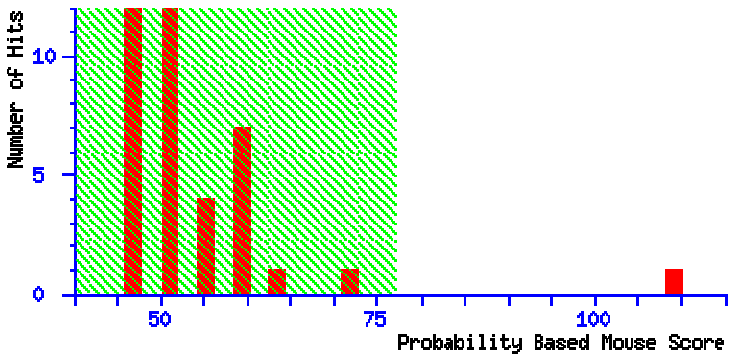
Sequence Coverage: **37%**

Matched peptides shown in **Bold Red**

1 MLLFFTSTIV NLFVIIRCNM NCKKIFITST LISLVSFLPG VSFSDVIQED
51 SSPAGSVYIS AK**YMPTASHF GK**MSIKEDSK **NTQTVFGLK**K DWDGVK**TPSS**
101 **DSGNNSIIFT EK**DYSFK**YEN NPFLGFAGAI GYSMNGPRIE FEVSYETFDV**
151 **K**NPGGNYKND AHMYCALDTG TPGSTQGATL NSSVMVKNEN LTDIALMLNA
201 CYDITLEGMP VSPYVCAGIG TDLVSVINAT NPKLSYQGK**L GISYSINPEA**
251 **SIFIGGHFHR** VIGNEFKDIT TSKIFTSTGK **LATAASPGFA SATLDVCHFG**
301 **IEIGGR**FVF

No match to: 656.0867, 842.5462, 1154.5780, 1782.0300, 1865.9820, 2211.2160, 2276.1990, 2291.2100, 3027.4900

Annex 18. Mascot search results from IDE8 spot 1 (Chapter 5)



Score: 109; MAP1-1 AAV54088 from Ehrlichia ruminantium

Nominal mass (M_r): **31192**; Calculated pI value: **6.84**

Fixed modifications: Carbamidomethyl (C)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **25**

Number of mass values matched: **9**

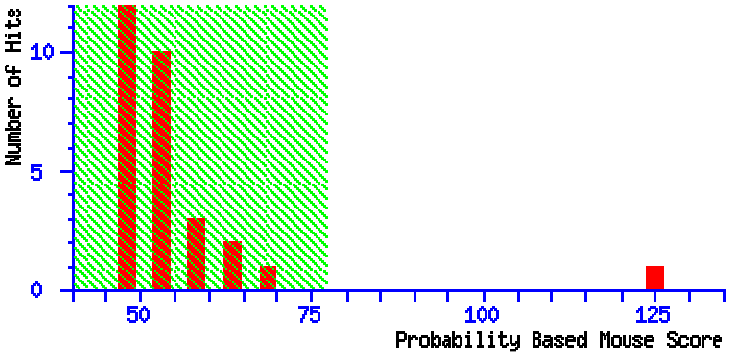
Sequence Coverage: **24%**

Matched peptides shown in **Bold Red**

1 MNYKKILVRS ALISLMSFLP YQSFAEPVSS NNIGNEXAK**E GFYISAKYNP**
51 **SIPHFRKFSA EETPVYGK**DS PTKKVFGLKK DGSITK**YSDF TR**TDISFEGQ
101 NNFISGFSGS IGYIMDGPR**V EIEAAYQK**FN PK**NPANETDT SDYYKHYGLS**
151 **R**AETMTDKKY VVLTNNGVTF SSLMFNACYD ITAEGVPFIP YACAGIGADL
201 ISIFDDINLK **FAYQGK**IGIS YPITPEISAF IGGYYHGVIG NKYNKIPVKL
251 PVTLTDAPQS TSASVTLDAG YFGGELGVRF TF

No match to: 744.4167, 841.1049, 842.5396, 1130.8780, 1132.1930, 1152.6210, 1154.5880, 1179.6560, 1192.5580, 1252.6670, 1268.6590, 1277.7300, 1473.7580, 1475.8110, 2240.2220, 2384.0400

Annex 19. Mascot search results from IDE8 spot 2 (Chapter 5)



Protein Score: 125; MAP1-1 AAV54088 from Ehrlichia ruminantium

Nominal mass (M_r): **31192**; Calculated pI value: **6.84**

Fixed modifications: Carbamidomethyl (C)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **18**

Number of mass values matched: **9**

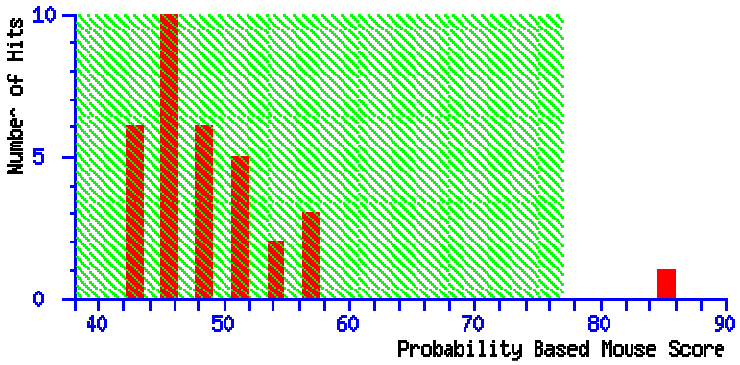
Sequence Coverage: **24%**

Matched peptides shown in **Bold Red**

1 MNYKKILVRS ALISLMSFLP YQSFAEPVSS NNIGNEXAK**E GFYISAKYNP**
51 **SIPHFRKFSA EETPVYGK**DS PTKKVFGLKK DGSITK**YSDF TR**TDISFEGQ
101 NNFISGFSGS IGYIMDGPR**V EIEAAYQK**FN PK**NPANETDT SDYYKHYGLS**
151 **R**AETMTDKKY VVLTNNGVTF SSLMFNACYD ITAEGVPFIP YACAGIGADL
201 ISIFDDINLK **FAYQGK**IGIS YPITPEISAF IGGYYHGVIG NKYNKIPVKL
251 PVTLTDAPQS TSASVTLDAG YFGGELGVRF TF

No match to: 744.4106, 1132.1930, 1134.0690, 1152.6140, 1168.5900, 1192.5590, 1252.6610, 1277.7270, 1472.7580

Annex 20. Mascot search results from IDE8 spot 3 (Chapter 5)



Score: 85; MAP1-1 AAV54088 from Ehrlichia ruminantium

Nominal mass (M_r): **31192**; Calculated pI value: **6.84**

Fixed modifications: Carbamidomethyl (C)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Number of mass values searched: **19**

Number of mass values matched: **7**

Sequence Coverage: **19%**

Matched peptides shown in **Bold Red**

1 MNYKKILVRS ALISLMSFLP YQSFAEPVSS NNIGNEXAK**E GFYISAKYNP**

51 **SIPHFR**K**FSA EETPVYGK**DS PTKKVFGLKK DGSITK**YSDF TR**TDISFEGQ

101 NNFISGFSGS IGYIMDGPR**V EIEAAYQK**FN PKNPANETDT SDYYK**HYGLS**

151 **R**AETMTDKKY VVLTNNGVTF SSLMFNACYD ITAEGVPFIP YACAGIGADL

201 ISIFDDINLK **FAYQGK**IGIS YPITPEISAF IGGYYHGVIG NKYNKIPVKL

251 PVTLTDAPQS TSASVTLDAG YFGGELGVRF TF

No match to: 823.1483, 825.1480, 841.1178, 842.5617, 994.2061, 1154.6200, 1268.6980, 1277.7690, 1355.7920, 1472.8170, 1517.7670, 2239.3330